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TITLE: The Influence of Primary Microenvironment on Prostate Cancer Osteoblastic Bone Lesion Development

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14. ABSTRACT

Loss of the stromal TGF-β signaling in the prostate has been shown to initiate prostate cancer (PCa), promote PCa progression, and facilitate the development of mixed osteoblastic/osteolytic bone lesions. However, the effects on bone lesions are found to be transient. We thus focused on delineating the context-dependent role of TGF-β signaling in the bone microenvironment effects on PCa bone lesions. Using genetic engineered mouse models, TGF-β signaling is cell-specifically knockout (KO) in the prostate fibroblasts and osteoblasts in the bone by *Tgfbr2*^{Col1CreERT} KO, or in the myeloid lineage cells, including osteoclasts in the bone by *Tgfbr2*^{LysMCre} KO. We found that PCa-induced bone lesions were significantly increased by *Tgfbr2*^{Col1CreERT} KO, but were significantly decreased by *Tgfbr2*^{LysMCre} KO, suggesting that osteoblastic TGF-β signaling inhibits PCa bone lesions development, but myeloid TGF-β signaling promotes PCa bone lesion development. In PCa patient bone metastasis samples, we found that the expression of TGF-β type II receptor (TβRII) was frequently lost in cancer-associated osteoblasts, but highly expressed in PCa cells. Thus we used the *Tgfbr2*^{Col1CreERT} KO mice to study the mechanism with the goal of identifying a druggable downstream mediator for PCa bone metastasis. Basic FGF (bFGF) was identified to mediate the effect of increased PC3 bone lesions by stimulating tumor cell proliferation, angiogenesis, and cancer-associated fibroblasts (CAFs) formation. *In vitro* studies showed that bFGF promoted osteoclastogenesis and inhibited osteoblastogenesis in a dose-dependent manner. bFGF had no direct effect on PC3 cell proliferation, suggesting an indirect effect of bFGF, possibly through increases of angiogenesis and CAFs formation. Furthermore, to determine the mechanism by which bFGF was up-regulated in osteoblasts with loss of TβRII, we found that the up-regulation of bFGF was concurrent with increases of PTH1R and pCREB in osteoblasts co-cultured with PC3 cells. Blocking PTHrP using neutralizing antibody could abolish these increases. Thus, bFGF is up-regulated at least partially through increased PTHrP signaling. The significances of our study are the determination of the role and mechanism of osteoblast-specific TβRII in PCa bone metastasis; Loss of TβRII results in increased PTH1R/pKA signaling, which up-regulates bFGF, making bFGF a promising therapeutic target in PCa bone metastasis by targeting both the PTHrP and TGF-β signalling that are pro-metastasis.

15. SUBJECT TERMS
TGF-β signaling, TβRII, *Tgfbr2*^{LysMCre} knockout, *Tgfbr2*^{Col1CreERT} knockout, bone lesions, bFGF, PTHrP, pCREB

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Introduction:

Based on our published and unpublished studies, we found that the primary tumor microenvironment effects are limited on early events of bone lesion development (*1*). Therefore, our current subjects are to study the influence of cell-specific TGF-β signaling in the bone microenvironment on prostate cancer (PCa) bone lesion development, and to identify the druggable downstream factor for PCa bone metastasis. TGF-β signals through the ligands binding with the TGF-β type II receptor (TβRII) (*2*). Using bone cell specific TβRII gene (*TGFBR2*) gene knockout mice, we found that TGF-β signaling in fibroblasts and osteoblasts inhibits, but TGF-β signaling in cells of myeloid lineage, such as osteoclasts, promotes PCa bone lesion development. In PCa patient tissue microarray, TβRII expression was frequently lost in the cancer-associated osteoblasts (CAOBs). Focused on the *Tgfbr2*^{Col1CreERT} knockout mice that have deletion of *TGFBR2* in osteoblasts, we found that the increased bone lesions were associated with increased tumor cell proliferation, angiogenesis, and cancer-associated fibroblasts (CAFs) formation. bFGF was found up-regulated in CAOBs and mediated the increased bone lesions in *Tgfbr2*^{Col1CreERT} KO mice relative to the *Tgfbr2*^{Flox} mice. Functionally, bFGF mediates PC3-induced bone lesion development through indirect stimulation of tumor cell proliferation, direct promotion of osteoclastogenesis and CAFs formation, as well as through direct inhibition of osteoblastogenesis. Furthermore, we found the increased bFGF was concurrent with increases of parathyroid hormone receptor (PTH1R) and downstream transcription factor pCREB in *Tgfbr2* KO osteoblasts with PC3 co-culture. These increases were abolished by blocking PTHrP in the co-culture system, suggesting that bFGF is at least partially up-regulated by PC3-derived PTHrP binding with the increased PTH1R and activating the downstream PKA/pCREB signaling. bFGF is a potential target for PCa bone metastasis.

Keywords: TGF-β signaling, *Tgfbr2*^{Col1CreERT} KO, *Tgfbr2*^{LysMCre} KO, osteoblasts, osteoclasts, bFGF, PTHrP, PTH1R

Overall Project Summary:

To determine the cell specific role of TGF-β signaling in the bone microenvironment effects on PCa bone lesions.

Osteoblasts and osteoclasts specific *TGFBR2* knockout mice, *Tgfbr2*^{Col1CreERT} KO and *Tgfbr2*^{LysMCre} KO respectively, were generated in *Rag2*$^{-/-}$ immune deficient background and

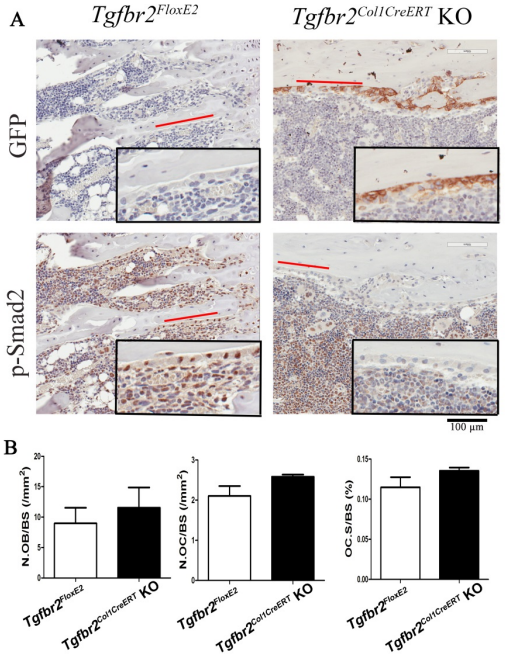

Figure 1. Osteoblast-specific *Cre* activity and bone histomorphometry analyses in *Tgfbr2*^{Col1CreERT} KO mice. Mice were i.p. injected with tamoxifen for 5 consecutive days at 4-5 weeks old, and the analyses were performed 5-7 d after the last tamoxifen injection. **A)** IHC of GFP and p-Smad2. Red lines indicate osteoblasts. IHC was done on serial sections of tibiae from *Tgfbr2*^{FloxE2} or *Tgfbr2*^{Col1CreERT} KO mice. GFP-positive cells (an indication of Cre activity) were specifically detected in osteoblasts of *Tgfbr2*^{Col1CreERT} KO mice, concurrent with the loss of p-Smad2 (scale bar, 100 μm). **B)** Histomorphometry analyses. Multinuleated (≥3 nuclei) TRAP-positive cells along the bone were considered as osteoclasts; osteoblasts were identified as being grouped, cuboidal or columnar in shape with a central nucleus, and localized on the bone surfaces. There were no differences in the numbers of osteoblasts or osteoclasts, or osteoclasts surface per bone surface analyzed between *Tgfbr2*^{FloxE2} and *Tgfbr2*^{Col1CreERT} KO mice (*t* test, two-tailed, $n \geq 3$).

crossed in mT/mG reporter mice. The *Tgfbr2*^{LysMCre} KO mice have been characterized (*3*). The *Tgfbr2*^{Col1CreERT} KO mice were characterized for the cell-specificity of Cre activities [(**Figure 1** (also see **Figure 2** in 2014 annual report)], and bones are phenotypically normal compared to the control floxed mice by x-ray and microCT analyses (data not shown)

PC3 PCa-induced osteolytic bone lesion development was significantly promoted in the *Tgfbr2*^{Col1CreERT} KO mice relative to *Tgfbr2*^{Flox} (control) littermates (see **Figure 1** in 2015 annual report). The same effect was repeated in DU145 cells (see **Figure 2** in 2015 annual report), and using *Tgfbr2*^{OCCre} KO mice. On the contrary, PC3 or LUCaP bone lesions were significantly decreased in the *Tgfbr2*^{LysMCre} KO mice relative to the *Tgfbr2*^{Flox} mice littermates (see **Figure 4&5** in 2015 annual report). The effects on PC3 bone lesions were shown in **Figure 2**. Further analysis of the bone lesions, we found the increased bone lesions were presented with increased angiogenesis and proliferation of the tumor cells in the bone (see **Figure 3** in 2015 annual report), and cancer-associated fibroblasts formation (**Figure 3**).

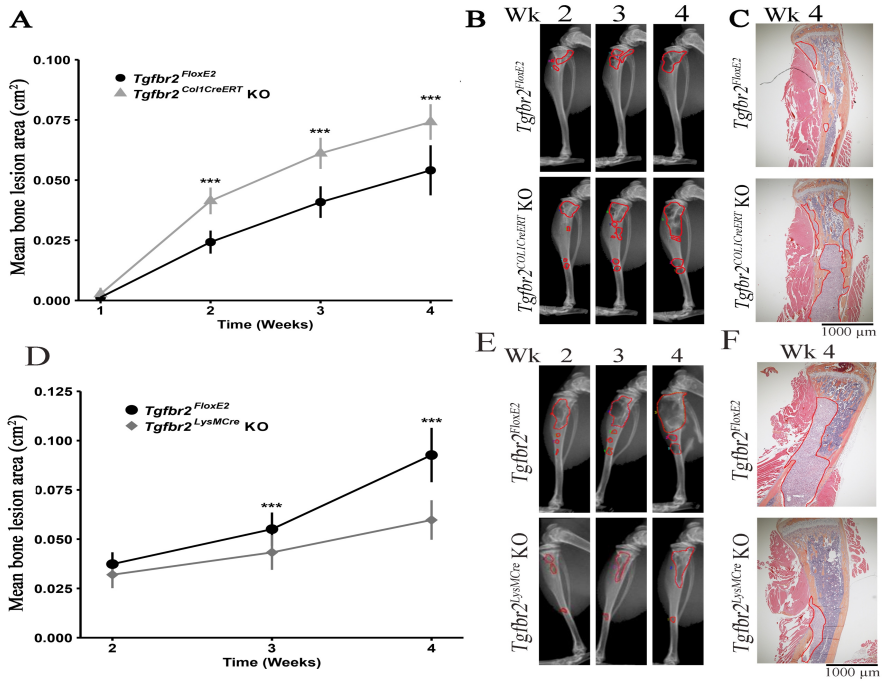

Figure 2. Effects of *Tgfbr2*^{Col1CreERT} and *Tgfbr2*^{LysMCre} KO on PC3-induced bone lesion development. **A** and **D)** Mean bone lesion area in PC3-injected mouse tibiae. The bone lesions were imaged by weekly X-rays, from which the bone lesion areas were measured using Metamorph software. **A**) The average total area of PC3 osteolytic lesions in *Tgfbr2*^{Col1CreERT} KO mice increased by 0.01 cm^2 per week more than lesions in *Tgfbr2*^{FloxE2} mice. Significant differences in bone lesion development were found between *Tgfbr2*^{FloxE2} and *Tgfbr2*^{Col1CreERT} KO mice (linear mixed-effects model with a random intercept, *** $P < 0.001$, $n \geq 9$). **D)** The average total area of PC3 osteolytic lesions in *Tgfbr2*^{LysMCre} KO mice was inhibited by 0.01 cm^2 per week relative to *Tgfbr2*^{FloxE2} mice. Significant differences in bone lesion development between *Tgfbr2*^{FloxE2} and *Tgfbr2*^{LysMCre} KO mice were found (linear mixed-effects model with a random intercept, *** $P < 0.001$, $n \geq 9$). **B and E)** Representative X-ray images of the osteolytic bone lesions at each time point. The circled lines show the regions of interest that were measured and analyzed using Metamorph Software. **C and F)** Representative H&E staining of the tibiae. The red outlines indicate the PC3 tumor growth in the bone marrow (scale bar, 100 µm).

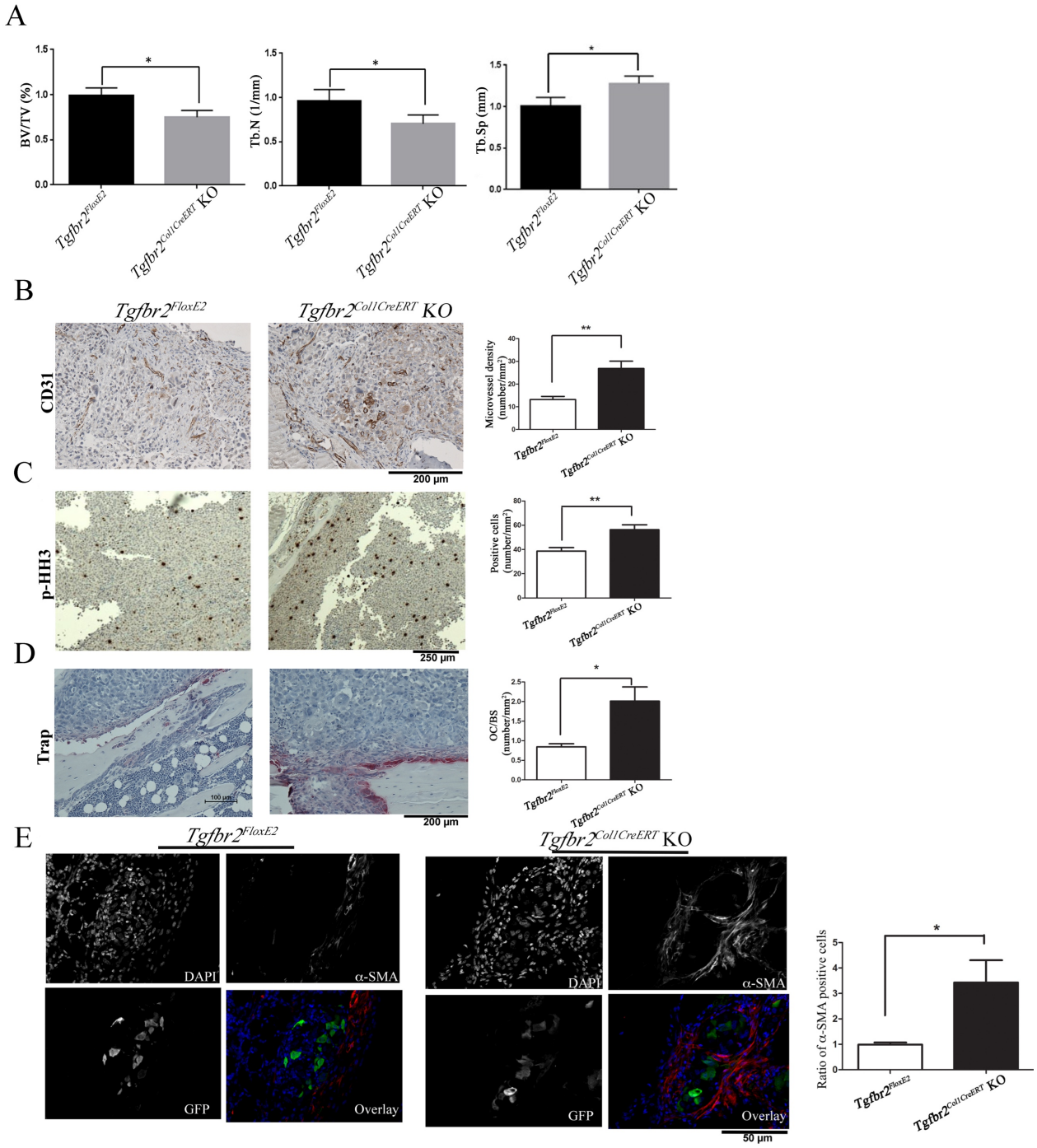

Figure 3 *Tgfbr2*^{Col1CreERT} KO promoted osteolytic lesion development and tumor growth.
A) Quantitative μCT analyses. Significant decreases in trabecular bone volume/total bone volume (BV/TV), trabecular bone number (Tb.N), but increased trabecular bone separation (Tb.Sp) were found in *Tgfbr2*^{Col1CreERT} KO tibiae, relative to tibiae from *Tgfbr2*^{FloxE2} mice. All data were normalized to the contralateral tibia. (*t* test, two-tailed, * $P \leq 0.05$, ** $P < 0.01$, $n \geq 3$). Representative IHC staining of **B)** CD31 (2.0-fold increase) (scale bar, 200 μm) and **C)** phosphorylated-histone H3 (P-HH3) (1.46-fold increase) (scale bar, 250 μm). **D)** tartrate-resistant acid phosphatase (TRAP) staining (2.38-fold increase) (scale bar, 200 μm). Quantifications are shown to the right of the image panels. **E)** Representative immunofluorescence and quantification of the α-SMA positive fibroblasts (3.47-fold increase). The data was normalized to the number of GFP-positive PC3 cells (scale bar, 50 μm).

Therefore, we conclude that TGF-β signaling in cells of the osteoblasts inhibits, but in cells

of the myeloid lineage promotes PCa induced bone lesions.

To determine the cell-specific TβRII expression in PCa patient bone metastatic tissues. To correlate with the data generated from our mouse model, we performed immunohistochemistry staining of TβRII in patient tissue microarray with PCa bone metastasis tissues. TβRII was highly expressed in PCa cells. The expression level of TβRII in PCa cells if higher in bone metastasis tissues relative to soft organ tissues (**Figure 4F**). However, frequent loss of TβRII was found in cancer-associated osteoblasts (CAOBs) (**Figure 4A-C**).

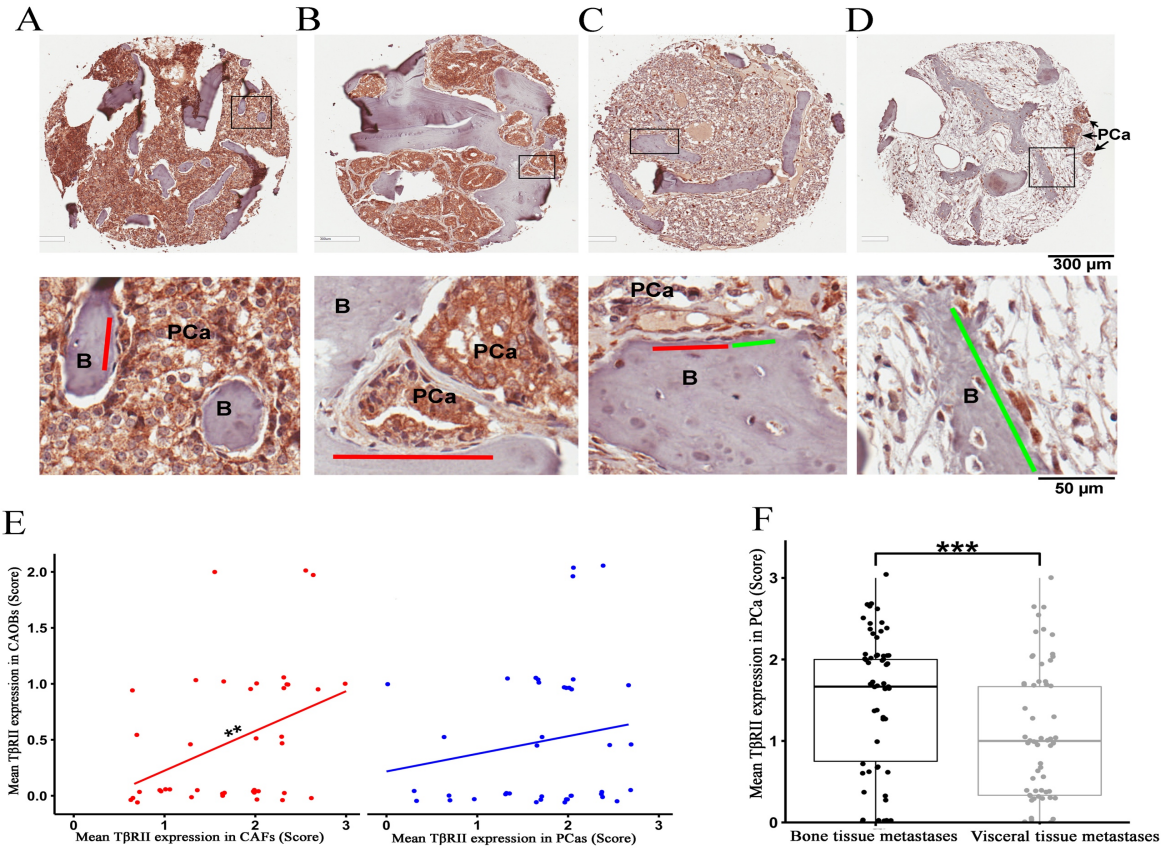

Figure 4. TβRII expression in PCa patient bone metastasis tissues.
Immunohistochemical (IHC) analysis of TβRII on a PCa patient bone metastasis tissue microarray containing 79 PCa bone metastasis and 62 visceral metastasis tissues (each having three cores) plus normal tissues as controls. **A-D)** IHC of TβRII showed positive (brown) staining in PCa cells in almost all samples examined. Loss of TβRII in all CAOBs was observed in 21 out of 40 tissues in which osteoblasts were identified (**A&B**, indicated by red lines); the estimated probability of this loss was 54.2% (95% CI, 36.3% to 68.2%). A mixture of both loss and expression of TβRII among CAOBs was observed in 16 out of 40 tissues (**C**, indicated by red and green lines); the estimated probability of this mixed expression was 40.5% (mixed-effects logistic regression, 95% CI, 27.6% to 54.4%). (scale bars: upper images, 300 μm; lower images, 50 μm) Expression of TβRII in all CAOBs was observed only in 3 out of 40 tissues (**D**, indicated by green line); the estimated probability of this expression was 4.5% (95% CI, 0.6% to 13.4%). **E)** Association analysis of cell-specific TβRII expression. TβRII expression in CAOBs was positively correlated with expression in CAFs, but not with expression in PCa cells (linear mixed-effects model, ** $P < 0.01$). **F)** TβRII expression in PCa cells of bone metastasis tissues was significantly higher than in visceral tissues (*t* test, two-tailed, *** $P < 0.001$).

To determine the downstream mediator for cell specific role of TGF-β signaling in the bone microenvironment effects on PCa bone lesion development.

bFGF was identified by cytokine array screening and confirmed to have increased expression at both mRNA level and protein level in PC3 induced tibiae of *Tgfbr2*^{Col1CreERT} KO relative to control mice (see **Figure 6&7** in 2014 annual report). IHC revealed that the bFGF was expressed mainly in osteoblasts and FGFR1 in PC3 cells, which were all increased in the lesions from *Tgfbr2*^{Col1CreERT} KO mouse bone relative to control mouse (see **Figure 6** in 2015 annual report).

Furthermore, we found that blocking bFGF using bFGF neutralizing antibody reduced the increased PC3 bone lesions in *Tgfbr2*^{Col1CreERT} KO, while the recombinant bFGF protein promoted the PC3 bone lesions in the control mice to the level as in the *Tgfbr2*^{Col1CreERT} KO mice (**Figure 5A, B&C**). These effects were correlated with respective changes in FGFR1, FGFR4, p-HH3 (phosphorylated histone H3, marker for cell proliferation), CD31 (marker for angiogenesis) and α-SMA (marker for CAFs formation) (**Figure 5D&E**).

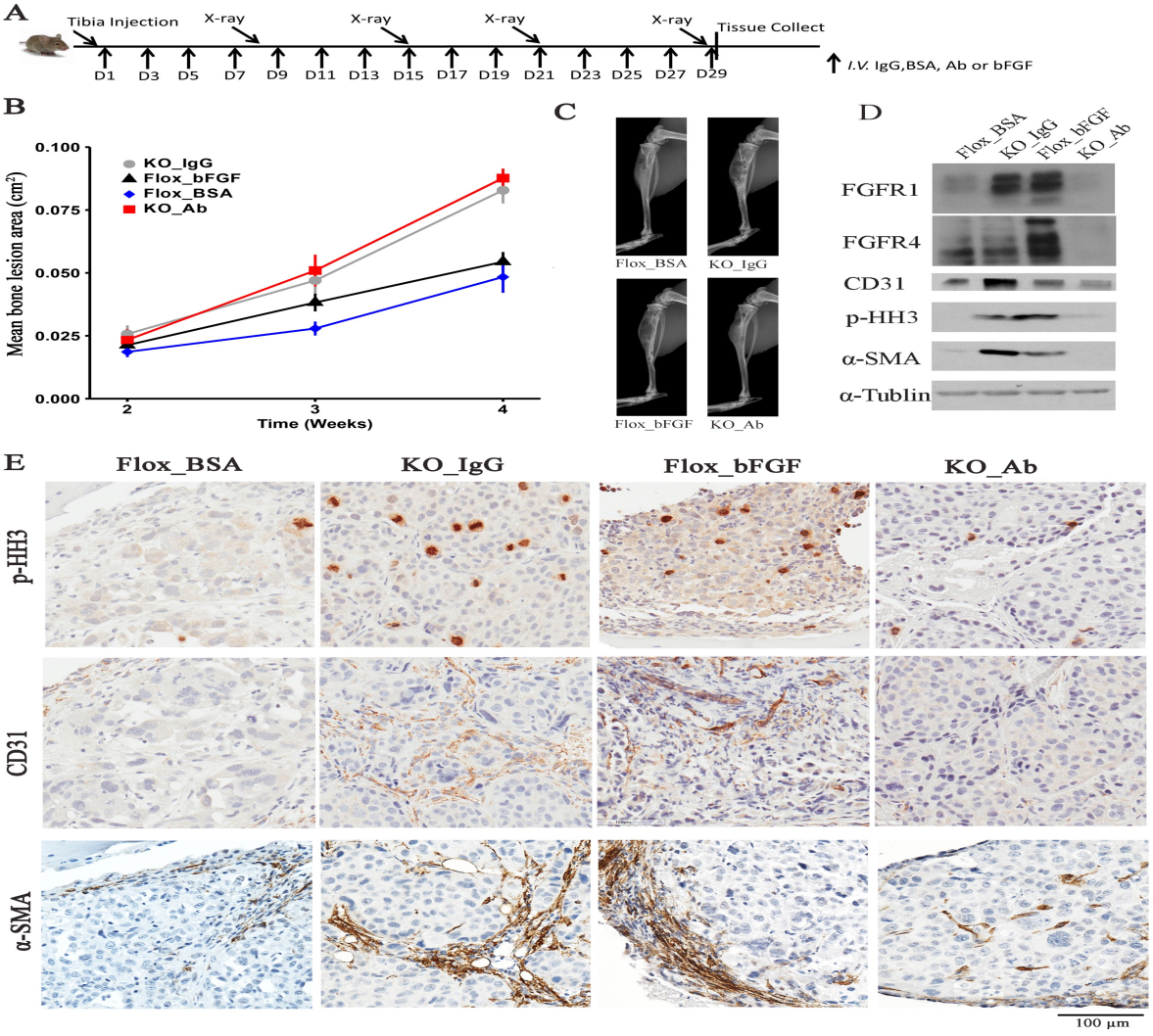

Figure 5. bFGF mediated the increased PC3 bone lesion in *Tgfbr2*^{Col1CreERT} KO mice.
A) Schedule of the drug treatment, X-ray image acquisition, and end-point collection. **B)** Quantification of mean bone lesion area in PC3-injected mouse tibiae. Significant bone lesion development was found between groups of the following: Flox_BSA vs KO_IgG, Flox_BSA vs Flox_bFGF, and KO_IgG vs KO_Ab (linear mixed-effects model with a random intercept, *** $P < 0.001$, $n \geq 3$). **C)** Representative X-ray images from each group at the final time point. **D)** Western blot analyses from whole tibiae harvested at the final time point. **E)** Representative images of IHC staining of p-HH3, CD31 and α-SMA (scale bar, 100 μm).

In vitro studies on bFGF effects on various cell types, we found that in a dose dependent manner, bFGF stimulates osteoclastogenesis. These effects were not influenced by paracrine osteoblast TGF-β signaling, since there were no differences between *Tgfbr2*^{Flox} and *Tgfbr2*^{Col1CreERT} KO group. bFGF inhibits ostoblastogeneses dose-dependently, and these effects were not influenced by cell-autonomous TGF-β signaling, as shown by no significant effects between control and *Tgfbr2* KO groups. However, bFGF has no direct effects on PC3 proliferation at 24 or 48 hrs treatments (**Figure 6**).

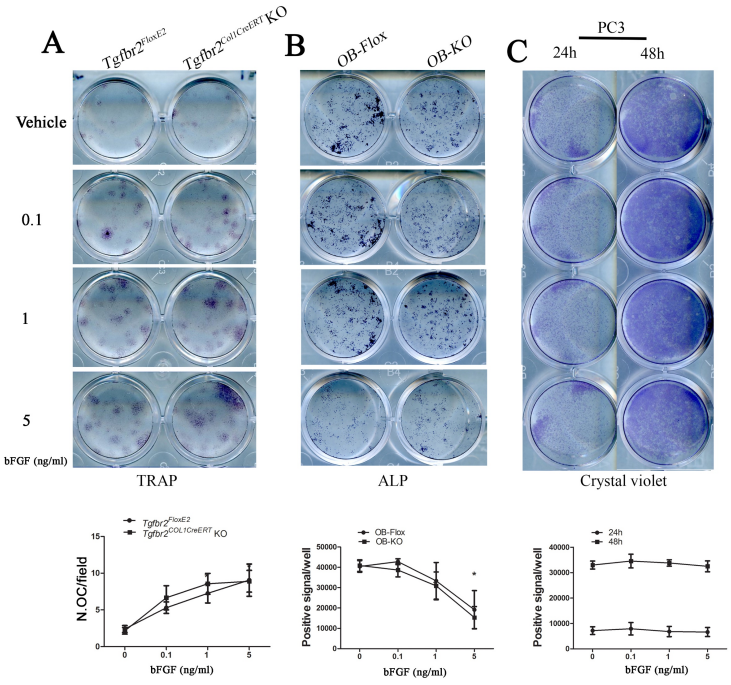

Figure 6. bFGF promoted osteoclastogenesis, inhibited osteoblastogenesis, but had no effect on PC3 proliferation.
Cells were exposed to bFGF at the doses and time points as indicated. **A)** Representative TRAP staining and quantification for osteoclasts from *Tgfbr2*^{FloxE2} and *Tgfbr2*^{Col1CreERT} KO littermates. A dose-dependent increase of osteoclast differentiation by bFGF was observed, but the effects of bFGF were not different between the cell lines. (two-way ANOVA, *** $P \leq 0.001$, $n \geq 3$). **B)** Representative ALP staining and quantification for control osteoblasts (OB-Flox) and osteoblasts with deletion of the *Tgfbr2* gene (OB-KO). A dose-dependent decrease of osteoblast differentiation by bFGF was observed, and the effects of bFGF were not different between the cell lines (two-way ANOVA, *** $P \leq 0.001$, $n \geq 3$). **C)** Representative images of PC3 cells proliferation that were stained with crystal violet. bFGF had no effect on PC3 cell proliferation after 24-h or 48-h treatment with various doses (one way ANOVA, $n \geq 3$).

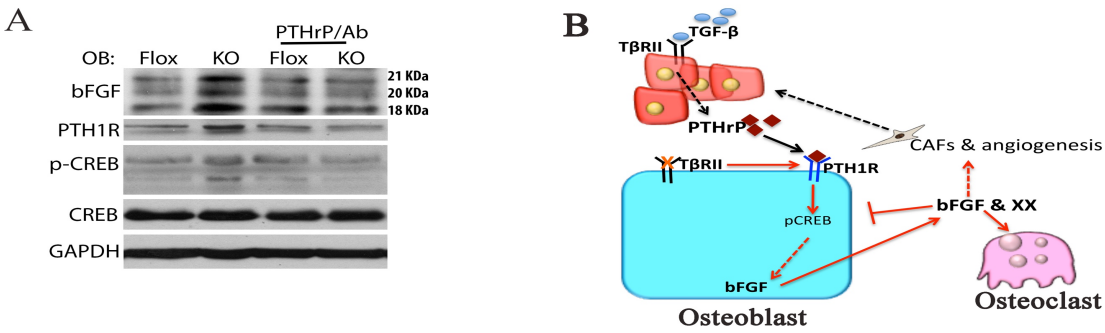

Figure 7. Loss of TβRII resulted an increase of PTH1R through which bFGF was up-regulated in osteoblasts by PC3-derived PTHrP. A) Representative western blots of total protein extracted from osteoblasts that were co-cultured with PC3 cells, n = 5. **B)** Loss of TβRII in cancer-associated osteoblasts (CAOBs) resulted an increase of PTH1R, possibly through disruption of the PTH1R/TβRII endocytosis induced by PCa-derived PTHrP. The increase of PTH1R was bound and activated the downstream PKA/pCREB signaling by PC3-drived PTHrP to up-regulate bFGF. bFGF, in turn, promoted PCa induced-bone lesion development by stimulating the differentiation of cancer-associated osteoclasts (CAOCs), inhibiting the differentiation of CAOBs, and potentially stimulates PCa proliferation through increasing cancer-associated fibroblasts (CAFs) and angiogenesis. In addition, high expression of TβRII in the bone metastatic PCa cells suggested an increased PTHrP expression by TGF-β from the bone microenvironment. Note: solid lines indicate our data and dashed lines indicate published data from others or our hypotheses.

Thus, we conclude that bFGF is a functional mediator for osteoblastic TGF-β signaling in PC3 bone lesion development. bFGF binds to FGFR1 and FGFR4 to regulate tumor cell proliferation, angiogenesis and CAFs formation.

To determine the mechanism by which loss of TβRII in osteoblasts up-regulates bFGF

Recent publication showed that TβRII could bind with PTH1R to undergo endocytosis in osteoblasts; loss of TβRII results an increase of PTH1R through disruption of the TβRII-PTH1R endocytosis (*4*). In osteoblasts that were co-cultured with PC3 cells, we did find that the up-regulation of bFGF was concurrent with the increased PTH1R and pCREB in *Tgfbr2* KO osteoblasts (relative to control osteoblasts). These increases were abolished by blocking PTHrP using neutralizing antibody in the co-culture system (**Figure 7**).

Thus, we conclude that bFGF is up-regulated in cancer-associated osteoblasts at least partially through PTHrP binding and activating the increased PTH1R, which is resulted from the loss of TβRII.

Key Research Accomplishments:

1. We found that TGF-β signaling in osteoblasts inhibited, but in myeloid lineage cells promotes PCa-induced bone lesion development.
2. We determined that the inhibited PC3 bone lesions by TGF-β signaling in osteoblasts were correlated with decreased tumor cell proliferation, angiogenesis and osteoclastogenesis in the bone.
3. We identified bFGF as a functional downstream mediator of the effect of osteoblastic TGF-β signaling on PCa-induced osteolytic bone lesion development.
4. We determined the mechanism by which bFGF facilitates bone lesions through promoting cell proliferation, angiogenesis and cancer-associated fibroblasts formation. In addition, bFGF stimulates osteoclastogenesis and inhibits osteoblastogenesis. Indirectly affects on PCa proliferation through angiogenesis and CAFs formation.
5. We found that the mechanism by which bFGF was up-regulated in cancer-associated osteoblasts with loss of TβRII was at least partially through the increase of PTH1R, which can be bound and activated by PCa-derived PTHrP.

Conclusions:

1. TGF-β signaling in cells of the osteoblast lineage inhibits, but in cells of the myeloid lineage promotes PCa induced bone lesions.
2. PCa patient bone metastasis tissues have loss of TβRII in cancer-associated osteoblasts, while high expression in cancer cells from bone metastasis tissues versus soft organ tissues
3. bFGF mediates loss of TGF-β signaling in the osteoblasts promoted PC3 osteolytic bone lesion development.
4. Coordination of PTHrP and TGF-β signaling in cancer-associated osteoblasts up-regulates bFGF
5. bFGF may be a better target inhibiting bone lesion development through effects on osteoblasts, osteoclasts and PCa cells.

Reportable outcomes:

Publications, Abstracts, and Presentations

a. Publications

Alexandra Vander Ark, Erica Woodford, Xiaohong Li*. (2016) Molecular mechanisms of therapies for prostate cancer with bone metastasis. *J. of Exploratory. research in pharmacology*. (Invited review) https://publinestorage.blob.core.windows.net/2764fac2-75cb-45ba-8d7d-61b1c0af3b92/jerp-16-23.pdf

Sourik S. Ganguly, Xiaohong Li and Cindy K. Miranti. (2014) The host microenvironment influences prostate cancer invasion, systemic spread, bone colonization, and osteoblastic metastasis. Front. Oncol., 4: 364-379. http://www.ncbi.nlm.nih.gov/pubmed/25566502

b. Manuscripts submitted for publication

Xiangqi Meng[1], Paul Daft[1], Alexandra Vander Ark[1], Jie Wang[1,2], Zachary Madaj[3], Galen Hostetter[4], Xiaohong Li[1*] TGF-β signaling in osteoblasts inhibits prostate cancer bone metastasis. *Submitted to Cancer Research*, 2016

Xiangqi Meng[1], Paul Daft[1], Alexandra Vander Ark[1], Jie Wang[1,2], Zachary Madaj[3], Galen Hostetter[4], Xiaohong Li[1*] Loss of TβRII in osteoblasts promotes prostate cancer bone metastasis through bFGF. 2016 SBUR Fall Symposium

Xiangqi Meng[1], Paul Daft[1], Sourik Ganguly[1], Alexandra Vander Ark[1], Jie Wang[1], Xiaohong Li*[1] TGF-β signaling in osteoclasts promotes, but in osteoblasts inhibits prostate cancer induced bone lesions. 2015 Cold Spring Harbor Laboratory Conference: Biology of Cancer: Microenvironment, Metastasis & Therapeutics.

Sourik S. Ganguly, Cindy K Miranti, Xiaohong Li* Increased Notch signaling and EMT transcriptional factors are associated with LuCaP23.1 osteoblastic bone lesions. 2014 SBUR Fall Symposium

Xiangqi Meng, Priscilla Lee, Xiaohong Li* TGF-β signaling in osteoclasts promotes, but in osteoblasts inhibits prostate cancer induced bone lesions. 2014 AACR meeting

c. Presentations

Poster presentation for 2014 AACR meeting (Dr. Xiangqi Meng)
Poster presentation for 2014 SBUR Fall Symposium (Dr. Sourik Ganguly)
Poster presentation for 2015 Cold Spring Harbor Laboratory Conference: Biology of Cancer: Microenvironment, Metastasis & Therapeutics (Dr. Xiaohong Li)
Podium presentation for 2016 SBUR Fall Symposium (Dr. Xiangqi Meng)

Other Achievements:

a. Awards and funding

1. 2014 AACR-Prostate Cancer Foundation Scholar-in-Training Award for Xiangqi (Neil) Meng
2. DoD postdoctoral training grant, 2016-2018, PI. Sourik Ganguly. Mentored by Xiaohong Li and Cindy Miranti
3. DOD idea award invited for submission, September 2015. "Claudin-19 activation in osteoblasts confers a growth-inhibitory bone microenvironment promoting prostate cancer dormancy" (not funded)
4. R01 submitted in June 2016. "PTH1R and TβRII in cancer-associated osteoblasts for bone metastasis" (not funded)
5. 2016 Fall SBUR Travel award for Xiangqi (Neil) Meng
6. DOD idea award invited for submission, October 2016. "TGF-β and PTHrP signaling in cancer-associated osteoblasts for prostate cancer bone metastasis" (Pending)

b. List of personnel (receiving pay from the research effort)

Xiaohong Li, Ph.D, Principal Investigator
Xiangqi (Neil) Meng, Ph.D, Post-doc fellow
Alexandra Vander Ark, M.S, Research technician

c. Mouse models generated

Tgfbr2^{Col1CreERT} KO, *Tgfbr2*^{OCCre} KO, *Tgfbr2*^{LysMCre} KO

References:

1. X. Li *et al.*, Loss of TGF-beta responsiveness in prostate stromal cells alters chemokine levels and facilitates the development of mixed osteoblastic/osteolytic bone lesions. *Mol Cancer Res* 10, 494 (Apr, 2012).
2. H. Ikushima, K. Miyazono, TGFbeta signalling: a complex web in cancer progression. *Nat Rev Cancer* 10, 415 (Jun, 2010).
3. X. Meng *et al.*, Myeloid-specific TGF-beta signaling in bone promotes basic-FGF and breast cancer bone metastasis. *Oncogene* 35, 2370 (May 5, 2016).
4. T. Qiu *et al.*, TGF-beta type II receptor phosphorylates PTH receptor to integrate bone remodelling signalling. *Nat Cell Biol* 12, 224 (Mar, 2010).

Appendices

frontiers in ONCOLOGY

REVIEW ARTICLE
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The host microenvironment influences prostate cancer invasion, systemic spread, bone colonization, and osteoblastic metastasis

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Prostate cancer (PCa) is the second leading cause of cancer death in men worldwide. Most PCa deaths are due to osteoblastic bone metastases. What triggers PCa metastasis to the bone and what causes osteoblastic lesions remain unanswered. A major contributor to PCa metastasis is the host microenvironment. Here, we address how the primary tumor microenvironment influences PCa metastasis via integrins, extracellular proteases, and transient epithelia-mesenchymal transition (EMT) to promote PCa progression, invasion, and metastasis. We discuss how the bone-microenvironment influences metastasis; where chemotactic cytokines favor bone homing, adhesion molecules promote colonization, and bone-derived signals induce osteoblastic lesions. Animal models that fully recapitulate human PCa progression from primary tumor to bone metastasis are needed to understand the PCa pathophysiology that leads to bone metastasis. Better delineation of the specific processes involved in PCa bone metastasize is needed to prevent or treat metastatic PCa. Therapeutic regimens that focus on the tumor microenvironment could add to the PCa pharmacopeia.

Keywords: prostate, cancer, tumor microenvironment, bone metastasis, EMT

FOREWARD

Prostate cancer (PCa), one of the most common non-skin cancers, results in the death of over a quarter million men annually worldwide (1) and 2.7% American men are estimated to die from this disease in their lifetime (2). The majority of PCa deaths are due to the development of metastatic disease, 80% of which is primarily localized in the bone (3). Furthermore, PCa induces an osteoblastic reaction within the bone, which is rarely observed with other bone-metastatic cancers.

For the patient who presents with metastatic disease, as evidenced by bone lesions detected by X-ray or it is suspected based on a high Gleason score, the first line of treatment after surgical removal of the primary tumor is androgen-deprivation therapy (ADT). The majority of prostate tumors require androgen for their growth and survival (4). Thus, the initial metastatic tumor burden in a patient can be essentially eliminated and they appear to enter remission. But the unfortunate fact is that some subpopulation of these cells either harbor or develop resistance to ADT and the tumor rapidly regrows.

Despite lack of evident dependence on circulating androgen, these castration-resistant tumors are still highly addicted to androgen and/or its cognate receptor, AR (5–8). Evidence for this is provided by several observations. First, second generation enhanced anti-androgen therapies, such as Enzalutamide, are effective, even if only for a while, in patients who failed the first round of ADT (5). Second, recent evidence indicates the tumor itself turns on androgen synthesis, so it no longer needs circulating androgen (9, 10). The successful use of drugs, such as Abiraterone (11), that target enzymes in the androgen synthetic pathway are also effective, again albeit for a short time, in patients who failed ADT. Third, the retention, mutation, and amplification of AR that accompanies ADT-resistant tumors indicate a heavy dependence on AR for the survival and continued persistence of these tumors. Several mutations in AR are known to confer enhanced function and include binding to other steroids or deletion of the ligand binding domain resulting in constitutive activation (12, 13). Fourth, AR could independently enhance invasion and metastasis through non-classical steroid receptor signaling mechanisms (14, 15). Currently, there are no approved therapies available that address these latter two events.

Development of additional therapeutic regimes to target metastatic tumors remains severely limited by the lack of knowledge about (1) what triggers PCa metastasis in the first place, (2) why it displays such a predilection for the bone, and (3) why it induces an osteoblastic bone phenotype. The molecular events thought to be involved in these three processes share a common theme; i.e., interactions with the host, often referred to as the tumor microenvironment. Current approved therapies are highly focused on targeting events occurring intrinsically in the tumor and do not fully consider the contributions of the host. Thus, better understanding of the host and tumor interactions that trigger and drive metastatic processes could provide additional avenues

for therapeutic intervention. In this review, we will discuss various possible strategies by which PCa cell interactions with the surrounding tumor microenvironment influence the development of metastases, homing toward the bone, and the development of osteoblastic lesions.

TUMOR MICROENVIRONMENT IN PROMOTING PROSTATE CANCER METASTASIS

INTRODUCTION

It is widely accepted that the tumor microenvironment, or stromal compartment, is biologically heterogeneous, consisting of various cell types, such as fibroblasts, endothelial cells, and immune cells, along with growth factors and cytokines, and numerous extracellular matrix (ECM) components. Paracrine signals from these factors released by the tumor activate signaling and gene expression in the neighboring cells and vice versa, ultimately setting up a cycle of reinforcement and continued signal propagation. Interactions between the cancer cells and this stromal compartment are required for invasion, angiogenesis, and metastasis of cancer cells to ectopic sites (16–18). The factors thought to drive the metastatic progression of PCa and to play an important role in the interaction of the tumor with its microenvironment are discussed below.

Olumi et al. (18), was the first to demonstrate the dependency of PCa development on the underlying fibroblasts. It was recognized that fibroblasts found near tumors, i.e., carcinoma-associated fibroblasts (CAFs), were fundamentally different from those in non-tumorigenic samples (19, 20). When CAFs isolated from human PCa patients were mixed with initiated human non-tumorigenic prostate epithelial cells, this was sufficient to initiate tumorigenesis. Normal fibroblasts lacked this capacity, implicating the importance of tumor-induced fibroblast effects feeding back on the initiated tumor cells. In another study, prostate stromal cells could replace Matrigel in LNCaP subcutaneous xenografts to promote tumor growth. One effect of the stromal cells was to promote angiogenesis (21). The stromal compartment of the normal prostate gland is full of smooth muscle cells. However, in PCa lesions, there was a dramatic loss of smooth muscle cells that were replaced by cells displaying myofibroblast characteristics, i.e., expression of Vimentin and increased production of matrix remodeling enzymes like Collagen I and Tenascin (22). This remodeling of the ECM and invasion of tumor cells into the surrounding stromal compartment define a cancerous lesion, as opposed to benign disease. The interaction of the prostate tumor cells with the remodeled matrix and the contribution of the tumor cells themselves to this process are critical first steps in the movement of tumor cells out of their normal niche.

INTEGRINS IN PCa PROGRESSION

Integrins are a large family of cell-surface glycoproteins, which form heterodimeric adhesion receptors. Integrins bind to a number of ECM components and regulate cytoskeletal organization to maintain cell shape and facilitate migration. These interactions also regulate cell survival, proliferation, adhesion, migration, and invasion (23, 24). PCa initiation and progression is accompanied by preferential expression of integrin α6β1, reduction in integrin α3β1, and complete loss of integrin β4 (25). Integrin α6, a laminin receptor is associated with poor patient prognosis and increased metastasis in a wide range of cancers (26, 27). In the normal prostate, integrin α6 complexed with integrin β4 is present at the basal cell/stromal interface; however, loss of integrin polarity occurs during progression of PIN to invasive cancer where basal cells are lost and integrin α6 complexed with integrin β1 abnormally appears in the luminal-like compartment (28).

Integrin α6β1 was shown to play two major roles in PCa, promoting cell survival and facilitating invasion and metastasis (28–30). Within the normal prostate epithelium, integrin expression is limited to the basal cells, being absent from the AR-expressing luminal cells. However, during PCa development, integrin α6β1 becomes co-expressed with AR in the tumor cells (31). AR directly binds the integrin α6 promoter and induces the expression of integrin α6β1, while simultaneously decreasing integrin β4 expression (29). Adhesion of PCa tumor cells to laminin to engage α6β1 promoted AR-dependent survival of the cells. Survival was mediated through AR-induced integrin α6β1 and subsequent activation of NF-κB and Bcl-xL expression (29). This AR/α6β1 pathway was active in metastatic cell lines, and elevated in castration-resistant cells. Laminins are abundant in the bone microenvironment, with Laminin-10 being the most highly expressed (32, 33), thus providing a mechanism for activating the integrin α6β1 survival pathway in the bone. Elevated NF-κB activity plays a critical role in PCa progression (34, 35). Another study demonstrated that secretory proteins from prostate neuroendocrine cells activate NF-κB signaling in the tumor cells, which in turn transcriptionally activates AR in the tumor cells to promote castration-resistant cell growth (36). The potential role of integrin α6β1 in this process remains to be determined. It was previously shown that the integrin β1a variant is expressed in PCa tumors, while the β1c variant is present in normal tissue (37). Signaling through the β1c variant was shown to suppress p27kip, a major negative cell cycle regulator and tumor suppressor known to be disregulated in PCa. Thus, a potential α6β1a variant may contribute to PCa proliferation.

Integrin α6 remains the primary integrin expressed in lymph node and soft tissue metastases, indicating high retention and selection for this integrin during metastasis (38). Cleavage of integrin α6 by uPA is associated with invasive PCa, the cleavage product is detected in tumors but not in normal prostate tissue, and promotes PCa cell invasion and migration on laminin (39, 40). Furthermore, inhibiting integrin α6-mediated adhesion or cleavage delayed experimental lung metastasis (28, 41), reduced bone growth in mouse femurs, and increased responses of metastatic PCa cells to ionizing radiation (42, 43).

Laminin integrins like α6β1 and α3β1 associate with transmembrane scaffold and membrane organizing molecules called tetraspanins (44). Tetraspanin KAI/CD82 was first identified as a metastasis suppressor in PCa (45). Loss of CD82 in human PCa correlates with poor prognosis, but by itself is not sufficient to predict metastasis (46). Studies in PCa cell lines *in vitro* demonstrate that CD82 is capable of suppressing integrin-based functions including signaling, migration, and invasion (47–50). CD82 regulates the internalization and turnover of integrins and suppress integrin signaling through Met and Src (47, 48). Its role in suppressing integrin functions was further validated in KO mice. However, the dependency on integrins for its metastasis suppressive functions did not prove to be valid (CKM, unpublished

data). Furthermore, while restoration of CD82 to metastatic cells suppresses metastasis, it loss in primary PCa is not sufficient to induce metastasis in genetically engineered mice (CKM, unpublished data). Thus, additional factors or as yet unidentified mechanism is involved in CD82 suppression of metastasis. A more likely target is its role in promoting cell–cell adhesion (51), discussed further in the EMT section.

The importance of integrin α2β1 in PCa metastasis is also emerging (31). Integrin α2β1 binds collagen, another major component of the prostate basement membrane and bone microenvironment. Manipulation of integrin α2 expression in LNCaP cells demonstrated a direct correlation between integrin α2 expression and the ability to grow in the bone (52). RANKL expression in PCa cells enhances bone metastasis. Integrin α2 integrin expression and function was stimulated in PCa cells overexpressing RANKL (53).

Thus, many studies support the importance of integrins in PCa development and progression by promoting, survival, proliferation, invasion, and metastasis. Targeting specific integrins and their matrix interactions may provide a way to prevent metastatic bone PCa.

PROTEASES IN PCa TUMOR INVASION AND METASTASIS

Breakdown of the basement membrane surrounding the prostatic ducts and invasion of prostate cells into the stromal compartment defines the pathology of prostate adenocarcinoma. Proteases that mediate basement membrane and stromal ECM degradation are crucial for this process and represent the first steps toward metastatic dissemination (**Figure 1**). Penetration into and out of the vasculature and lymphatics similarly requires proteases (54, 55).

Type II transmembrane serine proteases, like Matriptase and Hepsin, which are important for normal epithelial tissue development and repair, contribute to the breakdown of the basement membrane in PCa (56). Hepsin is dramatically up-regulated in PCa and represents one of the most highly overexpressed proteins in PCa microarrays (57). Elevated Hepsin expression is associated with high Gleason score and poor clinical outcome (55, 58, 59). Hepsin overexpression in the prostate epithelium causes disorganization of the basement membrane and its overexpression in PCa cell lines promotes lymph node metastasis (57). Hepsin reportedly cleaves and activates pro-HGF produced by activated fibroblasts within the stroma, thereby activating the receptor tyrosine kinase Met signaling pathway; a known activator of epithelial cell scattering, migration, and invasion (60). However, it was also reported that another Hepsin target is Laminin-5, a major component of the prostatic basement membrane, which is lost during PCa development (61).

Matriptase is expressed in many cancers and increased expression is seen in primary prostate tumors and metastatic lesions (62–64). One of its reported targets is Laminin-5, deposited in the prostatic duct basement membrane primarily by the basal epithelial cells. Laminin-5 expression is lost in PCa, coinciding with the

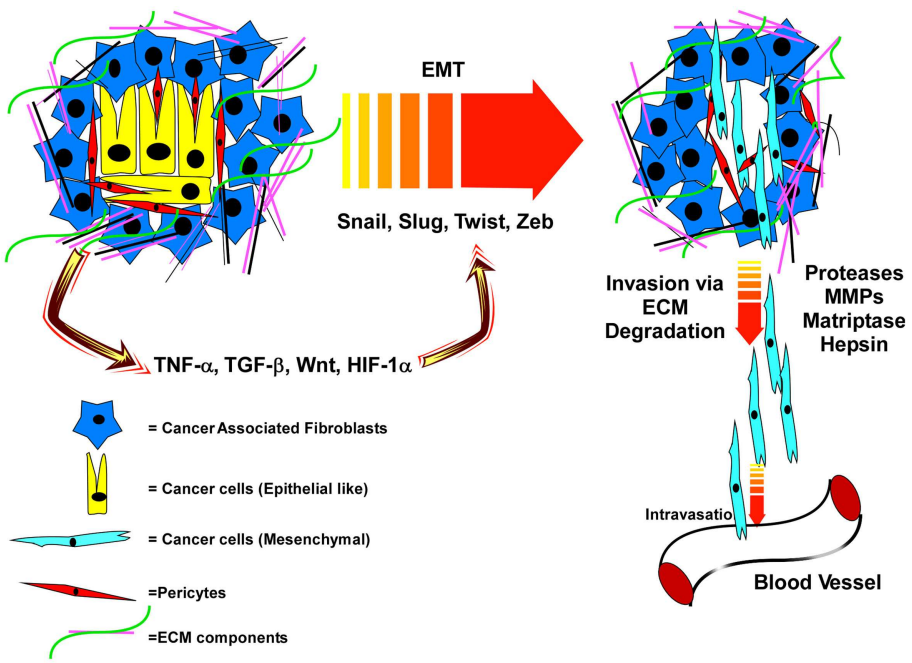

FIGURE 1 | Interactions of PCa cells with an extracellular matrix that is remodeled by cancer-associated fibroblasts, and soluble factors and proteases released within the tumor microenvironment induce EMT and subsequent invasion and dissemination of cancer cells. In the primary tumor microenvironment, the epithelial cancer cells are surrounded by the cancer-associated fibroblasts (CAF), pericytes, and various extracellular matrix (ECM) proteins. This tumor microenvironment produces various factors like TNFα, TGFβ, Wnt, and HIF-1α which promote EMT via up-regulation of specific transcription factors. EMT programing leads to a mesenchymal phenotype of the cancer cells and with the help of various proteases (MMPs, Matriptase, Hepsin), the cancer cells cleave the ECM, break away from the tumor microenvironment and intravasate into the blood vesicles to travel to distal organs.

loss of its primary receptor, integrin β4 (65). Active degradation combined with loss of secretion within the emerging PCa cells likely accounts for the lack of Laminin-5 in PCa tumors. Whether Laminin-10, another component of the prostate basement membrane, which is not lost during PCa development, is also cleaved by Matriptase or Hepsin is not known.

While Hepsin expression is apparently not controlled by AR, both Matriptase expression and its cleavage is highly controlled by androgen signaling (66). Rapid cleavage within minutes of androgen stimulation, mediated by Src signaling, is followed by a more long term increase in new Matriptase mRNA, thus providing a mechanism for replenishing depleted pools. Because Matriptase is essential for detachment of epithelial suprabasal cells during skin differentiation (67), and the AR-positive luminal cells also must detach upon differentiation, it is highly likely that Matriptase is also important in prostate epithelial differentiation. That AR, within the newly emerging luminal cells, can control integrin expression and a protease that degrades basement membrane has interesting implications about how AR contributes to preserving a luminal phenotype and may promote loss of basal cells during PCa development (68).

Two other proteases, which are known direct AR transcriptional targets, PSA and TMPRSS2, are normally secreted into the lumens of prostatic ducts (69, 70). However, due to loss of epithelial polarization and invasion into stromal areas, these enzymes are now also present within the tumor microenvironment (71). Their relative importance in PCa development or progression has remained largely undetermined.

The most extensively studied proteases linked with invasion and metastasis are the matrix-metalloproteinases (MMP). MMPs are involved in the degradation of the stromal ECM components such as Collagen and Fibronectin (72, 73). In normal tissues, MMPs play a major role in ECM remodeling involved in development and tissue repair. Their misregulation contributes to many disease states, including rheumatoid arthritis, pulmonary emphysema, and tumor invasion and metastasis (74–77). Active MMPs are secreted mainly from the cells in the tumor microenvironment, such as connective tissue, fibroblasts, endothelial cells, osteoblasts, macrophages, and neutrophils but also by cancer cells. Active MMPs are used by cancer cells to invade the stromal compartment both at the primary site and at metastatic sites (75, 76).

In PCa, MMP-2 and -9 are considered useful prognostic markers and these MMPs promote invasion and metastasis of PCa cells (78, 79). Elevated levels of MMP-2/9 in serum or plasma are correlated with high Gleason score (78–80). Overexpression of MMP-1 promoted PCa cell invasion and experimental metastasis, and inhibition of MMP-1 activity decreased PCa tumor growth in mice, indicating the importance of MMP-1 in regulating PCa invasion and metastasis (81). Induction of PCa invasion by MMP-9 is mediated through cleavage and subsequent inactivation of the serpin protease nexin-1 (PN-1). PN-1 is known to inhibit urokinase plasminogen activator (uPA) and thus inhibits PCa progression and metastasis (82). uPA and its receptor (uPAR) promote PCa metastasis, as down regulation of uPA or uPAR inhibited PCa cell invasion and metastasis (83, 84). When this is coupled with the reported role of uPA in cleaving integrin α6 (39), it becomes apparent how concerted efforts of proteases and integrin-based cell adhesion work together to promote invasion and metastasis.

Attempts to therapeutically target the MMPs, as a whole class, failed in clinical trials; resulting in worse outcomes (85). The lack of specificity to specific MMPs and the unforeseen protective role of some MMPs are thought to have contributed to the failure. Thus, there has been much resistance to trying to identify specific MMP inhibitors. On the other hand, preclinical testing of a small molecule Hepsin inhibitor demonstrated it blocked PCa metastasis in a genetic mouse model (57). Curcumin has the capacity to inhibit androgen induced Matriptase activation and displays anti-metastatic properties (86). Antibodies that block Matriptase cleavage have been reported (87), and a natural protein product produced by bacteria, Ecotin, is a natural Matriptase inhibitor (88), either of which may offer a therapeutic advantage. More work in defining the protease targets and mechanisms for inducing invasion and metastasis is clearly warranted.

EPITHELIAL-MESENCHYMAL TRANSITION IN PROMOTING PCa METASTASIS

The steps that lead to PCa metastasis (**Figure 1**) include degradation of the ECM, detachment of the cancer cells from the ECM as well as their detachment from each other, migration toward and subsequent entry into the blood or lymphatic system (89–91). The machinery and signaling pathways used in these invasive events are part of the normal wound healing response of epithelial tissues. Upon tissue damage, a host of growth factors and cytokines are released from the blood stream that activate the stromal fibroblasts (PDGF, TGF-β), endothelial cells (VEGF, FGF, IL8), and epithelial cells (HGF, TGF-β) to repair the tissue and fill in the wound (92). Through these signals epithelial cells are forced to loosen their matrix adhesions via activation of proteases and integrin signaling, and are induced to migrate across matrix being remodeled by the stroma. Some may even detach from each other to facilitate filling in the wound. It is this latter event, loss of cell–cell adhesion and depolarization of epithelial cells that is thought to trigger the final conversion of primary cancer cells into metastatic ones. Once loosened from the matrix and from each other, these cells are now free to roam if they have acquired the proper mutations that allow them to survive as non-adherent cells.

This process is referred to as epithelial–mesenchymal transition (EMT), where the loosened epithelial cells take on the physical properties of mesenchymal fibroblast-like cells (92). The classical marker of EMT is cadherin switching; where E-Cadherin expression is lost and N-Cadherin is gained (89, 90). E-cadherin, which promotes homotypic binding between two adjacent epithelial cells, prevents the cancer cells from breaking away from each other and reinforces tight junctions to preserve epithelial barrier function and apical/basal polarity (90, 93). The metastasis suppressor, tetraspanin CD82, promotes E-cadherin-based cell–cell adhesion, and suppresses integrin-based migration (51). Loss of this crucial regulator reduces cell–cell adhesion, while at the same time promoting enhanced migration; thus, its loss would strongly facilitate an EMT-like phenotype. Decreased CD82, E-Cadherin, or β-catenin (the anchoring protein for E-cadherin) is associated with poor PCa prognosis (23, 46, 94, 95). TGF-β is the most classical inducer of EMT, signaling through Smad family transcription factors to induce the expression of the EMT-regulating transcription factors Snail, Slug, Zeb-1, and/or Twist (96–100). These EMT-associated transcription factors, through interactions with

epigenetic regulators, control expression of genes involved in cell polarity, cell–cell contact, cytoskeleton structure, and ECM degradation (**Figure 1**), including repression of the E-cadherin gene (101).

While EMT is well-established in several other epithelial cancer types, its specific role in PCa remains controversial. This is complicated by the histological data demonstrating that PCa remains very epithelial-like in both primary tumors and in metastatic tissues; expressing E-cadherin and other classical prostate epithelial markers (94, 102). Yet, a few isolated tumor cell lines, especially those that have lost AR expression, readily adapt EMT phenotypes and are relatively invasive and metastatic (102–104). The necessary presence of AR in PCa tumors, which drives a luminal differentiation phenotype may make the conversion to EMT a relatively difficult process (103). It might explain the long latency for conversion of primary PCa to metastatic disease (105). The heterogeneous display of EMT phenotypes in PCa and other human tumor samples, has fueled the hypothesis that EMT is a transient process necessary for escape and dissemination, that reverses back, i.e., MET (mesenchymal epithelial transition) when cells reach their distant sites. A study that tested this hypothesis using a Tet-inducible model, demonstrated that transient induction of EMT was required for the migration of squamous carcinoma cells out of the skin into the blood stream, and subsequent shut off was required to establish metastatic tumors in the lung (106). If this happens in PCa is not clear, and the factors that drive it are even less clear.

The soluble factors that are secreted into a wound by physical disruption are the same factors present in the tumor microenvironment. Transforming growth factor-β (TGF-β) is a potent inducer of EMT and is released by many cellular components of the tumor microenvironment. However, the ability of primary tumors to respond to TGF-β is hindered by the normal growth inhibitory effects of TGF-β signaling, and thus the tumor cells typically block this pathway. So the cells must find ways to reactive some aspect of TGF-β signaling that doesn't cause growth suppression or find other indirect ways to stimulate EMT. One study suggested that direct cell–cell contact between tumor cells and platelets synergistically cooperated with TGF-β, to directly activate NF-κB signaling in the tumor cell to promote EMT and metastasis (107).

Inflammation induced during wound healing and in tumors also impacts the behavior of tumor cells. Many studies have demonstrated that wounding is a tumor-promoting event, especially chronic wounding where inflammation is high (92). The role of inflammation in PCa initiation was originally identified histologically by the presence of prostate inflammatory atrophy (PIA) in tumor samples (108). Recent mouse studies demonstrate that prostatic inflammation induced by prostitis, enhances basal-to-luminal differentiation and accelerates the initiation of PCa (109). Two sources for inflammatory signaling in PCa have been proposed, the stromal cancer-associated fibroblasts (110) or mesenchymal stem cells (111). A recent study highlighted the importance of adipocytes in inducing inflammatory responses in PCa cells within the bone through the lipid chaperone FABP4, triggering IL-1β expressing and oxidative stress protein HMOX-1 (112). Other studies proposed the inflammatory responses in PCa are mediated through NF-κB signaling (113). Inflammatory cytokines like TNFα and interleukins produced by both tumor cells and surrounding cells (114) activate NF-κB signaling and one consequence of this is the release of TGF-β (115, 116).

Non-TGF-β pathways can also activate EMT. TNFα can act independently of TGF-β to induce EMT by repressing GSK-3β, activating the AKT pathway, and stabilizing Snail (117–119). Wnt signaling also stimulates EMT in PCa cells. Expression of SOX2 induces EMT, and this was shown to be mediated by SOX2 binding to and activating β-catenin (120). The PCa-specific fusion and oncogene, TMPRSS2-Erg, enhances cell invasion (121, 122). Manipulation of the fusion gene in VCaP cells, altered Frizzled4 (Fzd4) expression (123). Fzd4 signaling promoted cell adhesion-related EMT phenotypes in VCaP cells. HIF1α, activated under hypoxic conditions, promotes aggressive tumor cell invasion and metastasis (124, 125). Overexpression of HIF1α in some PCa tumor cell lines promoted EMT that was dependent on β-catenin (126).

Further demonstration that EMT/MET phenotypic conversions are essential for the progression and metastasis of PCa to the bone is highly warranted. While current mouse models have been effective at defining specific molecular events occurring within the primary tumor or bone-resident tumors, the process whereby a confined prostate tumor is converted to a metastatic bone tumor has not been adequately modeled. The models do not currently reflect what is observed in human patients; i.e., AR-positive epithelial cells that migrate to and take up residence in the bone to induce an osteoblastic bone reaction. Understanding the specific genetic and epigenetic alterations that promote EMT-like phenotypes in PCa will be important to understanding the switch between indolent and lethal PCa, improving staging and prognosis of PCa patients and preventing over treatment.

TUMOR MICROENVIRONMENT IN PROSTATE CANCER BONE HOMING AND COLONIZATION

SECRETED FACTORS IN PCa BONE HOMING

Different types of cancers develop metastases in very specific organs. Several secreted factors have been proposed to promote organ-specific homing (**Figure 2**). A few of these chemotactic signals can attract cancer cells toward the bone. Conditioned media from osteoblasts differentiated *in vitro* served to induce migration and invasion of breast and melanoma cells, indicating osteoblasts secrete potent factors that can induce metastasis to the bone (127, 128). Osteonectin, a purified active factor from the bone, promoted invasion of bone-metastatic cancer cells, but not the non-bone-metastatic cancer cells indicating that bone possess chemotactic factors that can promote tissue-specific homing of cancer cells (129). It was demonstrated that osteoblast conditioned media containing higher amounts of TGF-β promoted chemotaxis and invasion of PC3 cells. Given the abundance of TGF-β in the bone environment, it could act as a chemo-attractant of PCa cells to the bone (130).

Chemokines and cytokines have chemo-attractant properties that play an important role in the cancer cell proliferation, survival, and gene transcription. Chemokine receptors are involved in many processes of cancer metastasis (131, 132). Mice bearing autoimmune arthritis have higher incidence of breast cancer

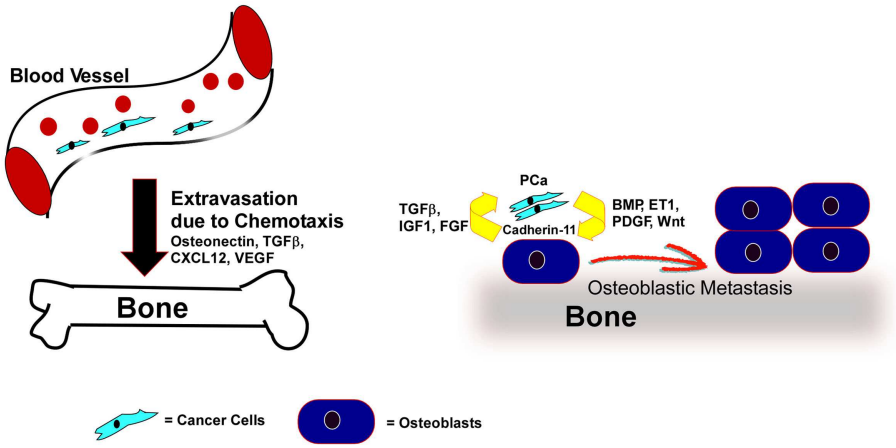

FIGURE 2 | PCa cells home to bone by chemo-attractants and colonize through direct association with osteoblasts, where the PCa cells secrete factors that promote osteoblastic responses and the osteoblasts reinforce tumor cell survival and growth. Expression of various chemo-attractants (Osteonectin, TGFβ, CXCL12, VEGF) guide PCa cells to extravasate and home toward the bone. Once in the bone-microenvironment the cancer cells interact with bone-forming osteoblasts via Cadherin-11. Factors like BMP, ET-1, Wnt, or PDGF, secreted from the cancer cells promote the proliferation and differentiation of osteoblasts. In turn the bone-microenvironment secretes soluble factors like FGF, IGF, and TGFβ to promote tumor cell survival and proliferation.

metastasis to the bone, which was proposed to be due to the presence of higher amounts of circulating levels of pro-inflammatory cytokines in these autoimmune arthritic mice (133).

The chemokine receptor, CXCR4, and its ligand CXCL12/SDF are widely studied in PCa bone metastasis (134, 135). In bone marrow, CXCL12 is expressed in osteoblasts, fibroblasts, and endothelial cells (136). Blocking CXCR4 in PCa cells using neutralizing antibody inhibited the dissemination and colonization of PCa cells in mice tibia following intra-cardiac injection (135). Akt1 reportedly induces the expression of CXCR4 in PTEN-null PCa cells, and overexpression of Akt-1 promoted intra-tibial tumor growth of PCa cells (137). These results indicate that Akt-1 might be inducing the CXCR4/CXCL12 axis and thus promoting PCa metastasis. PCa cells home toward areas in the bone marrow rich in osteoblasts where the hematopoietic stem cell (HSC) niche resides. In fact, PCa cells can bind to and displace mouse HSCs from the niche. Furthermore, the cancer cells egress out of the HSC niche into the blood when CXCR4 signaling is blocked by AMD3100 (138). These findings suggested that the CXCL12/CXCR4 axis is important for chemotaxis of PCa cells to the bone. However, inhibiting CXCR4 with CTCE-9908, a drug approved by FDA for osteosarcoma, inhibited spleen, liver, and lymph node metastasis of PCa cells, indicating CXCR4 may be a common metastatic factor, rather than one that is bone specific.

ECTOPIC SITE PRE-REMODELING

The famous "Seed and Soil" hypothesis, put forward more than 100 years ago by Dr. Stephen Paget, was used to explain why different types of cancer preferentially metastasize to different specific tissues. The theory proposed that distant organs, like the bone, provide a preferred "fertile soil" for cancer cells, and the cancer cells were preferentially attracted to that tissue. However, Isaiah Fidler's group demonstrated that tumor cells were present in vasculature of all the organs, yet metastasis only developed in certain organs but not in others (139, 140). David Lyden's group put forward the pre-metastatic niche model, where remodeling of only the preferred ectopic site(s) for metastasis occurs much earlier, before the cancer cells even break away from the primary tumor (139).

The niche remodeling events, mediated by soluble factors acting on non-cancer cells, govern the route of dissemination of cancer cells to a specific microenvironment. They demonstrated that bone marrow-derived hematopoietic progenitor cells expressing VEGF receptor 1 (VEGFR-1), homed to the specific metastatic sites through integrin α4β1. At the same time, the tumor cells secrete factors that induce the fibroblasts within the pre-metastatic niche to secrete Fibronectin, an α4β1 ECM ligand. The VEGFR1-positive cells then promote chemo-attraction and adherence of circulating tumor cells (141). Consistent with this idea, Hirutsuka et al. (142) demonstrated in a mouse model of melanoma metastasis that VEGF-A, TGF-β, and TNF-α released from the primary tumors induced the expression of chemokines in the lung parenchyma but not in other organs. In another study, persistent STAT3 activation was detected in distant organs such as the lung before tumor cell arrival. S1PR1–STAT3 up-regulation in tumor cells induced S1PR1–STAT3 at these distant sites and in myeloid cells. Ablation of STAT3 in the myeloid compartment inhibited STAT3 activity in the lungs, inhibited formation of pre-metastatic niche, and inhibited lung metastasis (143). Whether a similar pre-metastatic niche remodeling and non-tumor cell signaling governs PCa bone metastasis is not known. However, a past study demonstrated enhanced bone metastasis of orthotopic xenografted human cell lines upon androgen ablation (144). ADT in human PCa patients induces bone loss that is often corrected by bisphosphonates (145). Similarly, castrated mice lose bone mass (146). The full contribution of ADT to pre-metastatic niche conditioning, bone metastasis, osteoblastic reactions, or emergence of castration-resistant disease, as a result of bone-microenvironment interactions needs further investigation.

ADHESION MOLECULES IN PCa COLONIZATION

Several studies have suggested that the choice of metastatic organ is not necessarily dictated at the time tumor cells escape the tumor and end up in the circulation. Circulating tumor cells can be found in patients who do not have or do not develop metastatic disease (147). Furthermore, tumor cell entry is not restricted to specific organs, majority of circulating tumor cells extrude into tissues (147, 148). A PCa study demonstrated that 50% of the patients with primary tumors had circulating tumor cells as well as tumor cells lodged in the bone (149), yet only 12–13% of these patients ever develop metastatic disease. Thus, additional events are required for the tumor cells to grow and colonize the metastatic site.

Cadherin-11, commonly known as osteoblast cadherin, is primarily found in osteoblasts, with very low but detectable expression in brain, testis, and lung (150, 151). It is an adhesion molecule that mediates many steps of osteoblast maturation (150, 151). Cadherin-11 expression is increased in metastatic PCa compared to primary tumors but is not present in normal prostate tissue. Furthermore, elevated Cadherin-11 was found in human PCa bone metastasis relative to lymph node metastasis (152), indicating cadherin-11 is specifically associated with bone metastasis. Cadherin-11 may mediate the binding of cancer cells to osteoblasts (**Figure 2**). Such binding might also promote cross talk between cancer cells and osteoblasts and induce osteoblastic lesions. Intracardiac injection of PC3 cells expressing Cadherin-11-specific shRNA displayed a significant decrease in bone metastasis compared to the control cells (152). PCa cells derived from bone express high levels of cadherin-11, and expression of cadherin-11 in PCa cells promoted PCa cell invasion and migration and increased the adhesion and intercalation between osteoblasts in an *in vitro* culture model (153). Another group demonstrated that bone-tropic MDA-MB-231 breast cancer cells also express high levels of cadherin-11 compared to brain-tropic MDA-MB-231 cells. Thus, cadherin-11 is likely an important determinant of bone-tropism in cancer cells (154).

TUMOR MICROENVIRONMENT IN PROMOTING OSTEOBLASTIC LESIONS

Breast cancer metastasis is usually osteolytic (bone degrading); however, PCa is osteoblastic, i.e., leading to new bone formation (155, 156). It has been reported that some colon and cervical cancers are also osteoblastic (157). The exact mechanisms by which osteoblastic versus osteolytic metastases occur is still unclear. However, the differences are likely to reside in the differential interaction of tumor cells with the bone microenvironment. Bone, a dynamic connective tissue is constantly remodeling during an individual's lifetime. The process of remodeling is dependent on two cells types, osteoblasts and osteoclasts, both of which work in harmony to maintain the normal bone. Osteoblasts derived from mesenchymal stem cells in the bone marrow, make new bone. Whereas osteoclasts, which are modified macropages derived from monocytes, degrade bone (84). During osteoblastic metastasis, the bone remodeling favors bone formation over resorption.

FACTORS THAT INDUCE OSTEOBLASTIC BONE METASTASIS

The number of osteoblasts surrounding PCa cells is increased in osteoblastic bone metastasis (155). However, the newly formed bone is weak and fragile, lacking mechanical strength. These bones are composed of randomly orientated and loosely packed collagen bundles, resulting in weak bone strength and frequent fracture (158). Furthermore, the excessive bone growth disrupts the bone marrow compartment, reducing immune function. Many factors can induce the growth and differentiation of osteoblasts and their precursors (**Figure 2**). Tumor cells secrete many of these factors, and thus may actively promote osteoblastogenesis.

Bone morphogenetic proteins

Bone morphogenetic proteins are members of the TGF-β superfamily and various isoforms of BMP promote both prostate and breast cancer metastasis (159, 160). One major source of BMP expression appears to be from the tumor cells. Elevated expression of both mRNA and BMP-6 protein is detected in primary PCa tissues and in PCa cell lines (161, 162). BMPs could influence metastasis by acting directly on the tumor cells or through their effects on the bone microenvironment. For instance, BMP-2 induces resistance to apoptosis due to hypoxia (163) and promotes breast cancer cell invasion and migration (164); whereas, BMP-6 promotes migration and invasion of PCa cells (161). BMP-2 and -7 stimulated cellular migration and invasion of PCa cells (165), and BMP-6 acting through Smad signaling directly induced the transcription of extracellular proteases such as MMP-1 and 9, required for invasion (159).

Findings from genetically modified mice demonstrate that the normal role of BMPs in the bone is to induce the differentiation of osteoblasts (166, 167). For example, BMP-7 knockout mice have smaller skeletons and reduced mineralized bone (168). The ability of BMP-7 to control bone mineralization or osteoblast differentiation can be attributed to the induced expression of crucial differentiation factors, Runx2 and Osterix, in bone stromal cell precursors (169, 170). High BMP-7 expression was detected in PCa-induced bone lesions, while its expression in primary tumors is low (155, 171). However, its exact role in promoting osteoblastic vs. osteolytic lesions remains controversial. Nonetheless, BMP-7 produced by tumor cells has the potential to impact osteoblast differentiation within PCa bone lesions. The most intriguing aspect of BMP-7 is that while its expression is controlled by androgen and it is required for normal prostate development, the most elevated BMP-7 expression was observed in castration-resistant tumors within the bone (171). However, BMP-7 expression in prostate bone tumors appears to be largely growth suppressive and may promote PCa cell dormancy (172).

Elevated BMP-6 expression is also associated with PCa bone metastasis (159). BMP-6 produced by cancer cells was able to induce mineralization of M3T3 pre-osteoblasts, and blocking BMP-6 activity reduced osteolastic lesion formation by LuCaP 23.1 cells *in vivo* (161). Two studies demonstrated that Wnt5a or Wnt3a, generated by bone stroma cells, induces the expression of BMP-6 in PCa cells (173, 174). This was mediated by non-canonical JNK and canonical β-catenin signaling pathways, respectively (174). The induction of BMP-6 by Wnt5A secretion occurred in the absence of androgen and promoted androgen-independent growth of the tumor cells (173). Interestingly, in a recent patient-derived bone xenograft model, transplantation of human bone-metastatic

tumors into the bone, but not in the skin, resulted in castration-resistant tumor growth (175). Thus, the bone microenvironment has a high capacity to influence PCa treatment. The BMPs secreted by PCa cells through Wnt signaling, in turn induce the differentiation of osteoblasts. For instance, BMP-4 produced by PCa cells promoted osteoblast differentiation of mouse stromal cells, as measured by the production of alkaline phosphatase, osteocalcin, and collagen type II. At the same time, BMP-4 also stimulated the production of sonic hedgehog (Shh) by the tumor cells (176). Shh stimulated Smad1 and BMP receptor expression in the mouse stromal cells, which enhanced their response to BMP-4. The ability of Shh to induce osteoblast differentiation was promoted by collagen production and was Gil1-dependent, but Runx2-independent (177, 178). Thus, BMPs and Shh cooperatively provided cues for the growth of PCa cells and the differentiation of bone stromal cells. All these results indicate that BMPs and Shh from the PCa cells play an important role in inducing osteoblast differentiation from bone stromal cells, and thus likely contribute to osteoblastic bone phenotypes.

Endothelin 1

Endothelin 1, a potent vasoconstrictor along with other family members ET-2 and ET-3, is produced by the vascular endothelium. Plasma levels of ET-1 are elevated in several types of cancers, including PCa (179). ET-1 inhibits PCa cell apoptosis via enhanced Bcl-2 family member expression and PI3K/Akt activation (180). In ovarian carcinoma cell lines, ET-1 promotes invasion via the activation of extracellular proteases like MMP-1 (181). In addition to promoting cancer metastasis, ET-1 promotes osteogenic properties; ET-1 null mice exhibit hypoplasia of facial bones (182). ET-1 is a potent mitogenic factor of osteoblasts and patients with osteoblastic bone lesions have increased serum levels of ET-1 (171, 183). Elevated ET-1 might contribute to osteoblast proliferation and differentiation (184–186).

In clinical trials, atrasentan (endothelin A receptor antagonist) suppressed bone remodeling in castration-resistant metastatic patients (187). However, atrasentan in combination with docetaxel, a chemotherapeutic agent, did not improve progression-free survival in castration-resistant bone-metastatic patients (188). One of the potent pathways by which ET-1 promotes osteoblast activity is through the activation of Wnt signaling via the inhibition of the Wnt suppressor DKK1 (189). ET-1 inhibited DKK-1 expression, but also increased the expression of Type 1 collagen, a predominant protein constituent of bone matrix (185, 189).

Wnt

Wnts constitute a family of 19 secreted glycoproteins, whose dysregulation plays an important role in the progression of many cancers including breast, gastric, prostate, melanoma, and glioblastoma (190, 191). Wnt ligands bind to a seven-pass transmembrane receptor composed of Lrp5/6 and frizzled genes to transduce signals to the cytoplasmic protein Disheveled (Dsh). Dsh blocks GSK-β to inhibit β-catenin phosphorylation, by disrupting the β-catenin/Axin complex, leading to β-catenin stabilization, and its nuclear translocation to interact with TCF/LEF transcription factors (192, 193). Loss of APC activates the Wnt pathway by stabilizing β-catenin. Loss-of-function of APC mutations is common in many cancers (193–195). Increased nuclear β-catenin expression correlated with advanced, metastatic, and hormone-refractory prostate carcinoma (196). Upregulation of the Wnt pathway by means of increased Wnt secretion, decreased expression of inhibitors such as APC, sFRP, DKK1, or Wif1, or constitutive activation of β-catenin, induces the activation of downstream target genes like c-Myc, c-Jun, and various other genes important in both cancer development and metastasis (91, 192, 193). MMP-14, which promotes invasion and metastasis of cancer cells, is also a direct target of β-catenin/TCF signaling (197). The exact mechanisms that lead to elevated β-catenin in PCa are not clear, but it is not usually due to APC mutation (194).

Within the bone, Wnt signaling promotes osteoblast differentiation by directly stimulating Runx2 expression in osteoblasts both *in vivo* and *in vitro* (198). Wnt signaling also stimulates BMP-2 expression, inducing the trans-differentiation of non-osteogenic cells into osteoblasts (199). Wnt signaling may contribute to the osteoblastic phenotype. Blocking DDK1 expression in PC3 cells, which releases the block on Wnt signaling, switched the normal osteolytic phenotype induced by PC3 cell to osteoblastic. Conversely, overexpressing DKK-1 in C4-2B cells converted the normal mixed lesion to an osteolytic lesion (200). Thus, Wnt signaling contributes to PCa osteoblastic bone lesions. Further investigations in the specific components of the Wnt pathway involved, and determining if they also contribute to metastasis *per se* will be important.

Platelet-derived growth factor

Platelet-derived growth factor, a potent growth factor which plays an important role in tumor progression, consists of disulfide-bonded homodimer polypeptide chains of A, B, C, D, and heterodimer AB (201). Aberrant signaling through PDGF receptors promotes progression of many tumors, including PCa. PDGFRβ, which is frequently activated in bone-metastatic PCa patients, is activated both by PDGF-B and PDGF-D (202). PDGF-D, which promotes PCa cell proliferation and tumor growth, is overexpressed in prostate tumors with increasing Gleason score (203). Furthermore, PDGF also stimulates the interaction of PCa cells with bone stromal cells. In the bone microenvironment, PDGF is synthesized by platelets, macrophages, osteoclasts, endothelial cells, and all cells differentiating from mesenchymal stem cells, including pericytes, and osteoblasts (204). Thus, PDGF has the potential to act as a central connector for many interactions within the bone microenvironment that influence tumor growth. Blocking PDGF receptor signaling inhibits the growth of human breast and pancreatic cancer in bones (205, 206), and subsequently reduces bone resorption. However, a phase I clinical trial with a potent PDGFR inhibitor, imatinib mesylate in combination with docetaxel, in castration-resistant PCa patients with bone metastasis did not show any improvement in the median progression-free survival of patients, as compared to docetaxel alone (207). However, imatinib mesylate inhibits many other kinases, like Abl and c-Kit (208). Whether PDGFR inhibitors in combination with the second generation anti-androgens, Enzalutamide or abiraterone, will be more effective should be investigated.

FACTORS RELEASED FROM THE BONE MICROENVIRONMENT

Transforming growth factor-β

Transforming growth factor-β is one of the most abundant cytokines which induces osteoblast proliferation but inhibits its differentiation (209, 210). The effects of TGF-β on osteoclasts are controversial. Most of the studies investigating TGF-β in cancer bone metastasis have focused on osteolytic bone metastasis of breast and cancers other than PCa. A "vicious cycle" model has been proposed to explain TGF-β signaling and osteolytic bone metastasis. TGF-β, signaling through Gil2, in a Hedge Hog-independent manner, stimulates the expression of parathyroid hormone-related protein (PTHrP), which activates osteoclasts (211). In turn, more TGF-β is released after bone resorption, which further enhances cancer growth and osteoclast activation, initiating a "vicious cycle" (212, 213). Inhibition of TGF-β decreases osteolytic lesions induced by breast and melanoma cancer cells in mouse tibia (214–216). In the first report on the role of TGF-β in PCa bone metastasis, it was found that loss of TGF-β responsiveness in the fibroblasts induced the up regulation of CXCL16 and CXCL1, which promoted PCa cells adhesion to the bone matrix, and promoted mixed (osteoblastic/osteolytic) metastatic lesions (217). Thus, it will be important to further investigate the cell-specific role of TGF-β in the bone microenvironment on PCa osteoblastic bone metastasis.

Insulin-like growth factor

High serum levels of insulin-like growth factor are associated with higher risk of breast, prostate, and colorectal cancer. Signaling through IGF1R promotes cell proliferation, apoptosis, and invasion of cancer cells; which are all integral steps in cancer metastasis (218). For example, inhibition of IGF-1R diminishes the invasion of PCa cells and also inhibits expression of MMP-2, an extracellular protease necessary for invasion (219). The ability of IGF-1 to induce PCa cell proliferation and survival is dependent on loss of Pten, a tumor suppressor commonly lost in PCa (220). However, IGF is another important coupling factor in the bone, activating both bone formation and resorption. IGF, which promotes proliferation, invasion, and metastasis of cancer cells is released during bone resorption (84). One interesting study showed that neutralizing antibody to IGF, but not antibody to TGF-β, FGF, or PDGF, blocked the breast cancer anchorage-independent growth induced by resorbed bone extract; further supporting a unique role for IGF-1 in bridging the cross talk between the bone microenvironment and the cancer cells (221). Bone-derived IGF promoted bone metastasis of breast cancer cells by stimulating proliferation and inhibiting apoptosis of cancer cells (221). Elevated IGF-1 receptor expression in the stroma surrounding clinical PCa samples correlates with high Gleason score (222). Thus, IGF-1 released from the bone could stimulate the stroma to support PCa growth. Indeed, blocking IGF-1 and IGF-1 receptor inhibits PCa growth in the bone and reduced the osteoblastic bone formation. Thus, IGF-1 signal inhibition could be strategy for limiting PCa bone metastasis.

Fibroblast growth factor

Fibroblast growth factor, a family of ubiquitously expressed and secreted factors, regulates processes like development, wound healing, and neoplastic transformation through mitogenesis and angiogenesis. Increased expression of some growth factors from the FGF family and their receptors are reportedly associated with PCa progression. Aberrant activation of FGF receptors (FGFR) induces the activation of downstream targets like PI3K, MAPK, and STAT3, all of which play an important role in the progression of PCa (223–225). One of the FGF family members, FGF-1 (acidic FGF) is expressed in a majority of PCa and its expression is associated with high Gleason score (226). Levels of FGF-2 (basic FGF) and FGF-6 are also elevated in PCa tissues as compared to normal (227, 228). In one transgenic mouse model, FGFR1 overexpression led to PIN (229), but in another model, activated FGFR1 promoted adenocarcinoma and metastasis to lymph nodes and liver (230). FGF may promote PCa metastasis via regulation of cell survival (231). PCa bone metastases express FGF-8 and/or FGF-9, and both of these FGFs are reported to promote osteoblast differentiation and new bone formation (232–234). FGF-9 expression is also associated with high Gleason score and neutralizing antibody against FGF-9 inhibited bone formation and bone lesions in mice (232, 235). On the other hand, other family members, like FGF-7 and FGF-10, are required for normal differentiation of normal prostate epithelial cells (236). Nonetheless, overexpression of FGF-7 in a transgenic mouse prostate epithelium led to PIN (237), and overexpression of FGF-10 in the mesenchyme in the prostate regeneration mouse model was sufficient to induce multifocal PIN had low-grade PCa (238). Thus, the context and level of FGF signaling may differentially impact PCa development and progression. The mechanisms that underlie the differential expression of FGF members and their respective receptors to promote or inhibit PCa growth in different microenvironments, and the cellular constituents upon which it acts need to be better resolved in PCa.

CHALLENGES THAT REMAIN

The high dependence of host environmental factors on metastatic processes necessitates the use of animal models to clarify and demonstrate that dependency. The most commonly used model is the mouse, which has a physiology and prostate organ structure that is significantly different from human. Nonetheless, valuable insight can be gleaned from the proper models. Unfortunately, for PCa researchers many of the current mouse models fail to fully recapitulate human disease progression; i.e., an AR-dependent tumor growing in the prostate gland that spontaneously metastasizes to the bone to from an AR-dependent osteoblastic cancer.

Models using genetic introduction of known PCa-associated mutations into the mouse genome, rarely metastasize to the bone. The few models that do are neuroendocrine or use mutations not reported to be present in human disease. Nonetheless, one unifying theme in the models that do produce significant metastases is the abrogation of p53. While p53 loss is reported in <20% of metastatic PCa tumors, other mutations that indirectly alter p53 function remain a strong possibility. Another tumor suppressor, Pten, whose homozygous loss strongly correlates with metastatic progression in human cancer, but whose loss alone in the mouse does not lead to metastatic bone disease, may also contribute to metastasis. Interestingly, both p53 and Pten impact chromosomal stability (239), disruption of which is thought to be necessary

for the selection of genetic variants within the tumor that eventually evolve the capacity to metastasize. Recent studies suggest that EMT-associated transcription factors suppress p53 signaling and impact DNA repair; thus, linking disease progression by the tumor microenvironment with chromosomal instability (106). Identifying the factors that specifically lead to chromosomal instability, the genetic alterations within PCa that promote metastasis, and how the tumor microenvironment influences this, has the potential to significantly increase our understanding of metastatic conversion in PCa.

Several metastatic PCa cell lines isolated from bone metastases, either from human samples or mouse xenografts (VCaP, C4–2, PC3), retain high capacity to grow when implanted directly into the bone, or in a few cases when injected into the heart. Two PCa lines, one isolated from the brain, DU145, and one from ascites, ARCaP, can also grow when implanted in bone; however, the LNCaP line isolated from a lymph node grows poorly in the bone. Most of them, when implanted orthotopically in the prostate, do not metastasize to the bone; though some make it to the lung. ARCaP was reported to metastasize to the bone after orthotopic injection (240). This was accompanied by EMT conversion. Only a few of these, when implanted in the bone, make osteoblastic lesions (DU145, C4–2, ARCaP). Thus, whatever properties these cells once had in the human host that permitted their full metastatic progression in humans has been lost. Furthermore, at least 3 of these lines, PC3, DU145, and ARCaP express AR at such low levels they do not use the AR-regulated pathways seen in over 90% of human PCa metastases. The LuCaP series of human xenografted and SubQ-passaged tumors (241), isolated primarily from soft tissue metastases and still expressing AR, can grow and form osteoblastic lesions when implanted in the bone. Removal of primary LuCaP tumors following orthotopic injection, allowed the development of micrometastases to lymph nodes and soft tissues (242), but not bone. Thus, these studies indicate that many PCa tumor cell lines, whether they originally came from bone or not, have a high capacity to grow in bone. However, they all lack the ability to home to bone from the prostate, and their capacity to induce osteoblastic lesions is variable. Developing human lines or mouse models that can display the full metastatic progression, either through selection or genetic manipulation, remains the Holy Grail for understanding PCa metastasis and having models that can be used for effective therapy development and testing.

CONCLUSION AND PERSPECTIVES

Metastasis is the major cause of PCa death. Understanding how cancer cells metastasize toward the bone is needed to design drugs that prevent or interfere with PCa metastasis. Many studies suggest that EMT transcription factors drive the initial phases of PCa metastasis, although the events, either within the tumor or contributed by the tumor microenvironment, that trigger EMT specifically in PCa are still not known. Overexpression of EMT transcription factors, in conjunction with responses to host-derived chemotactic factors, might lead to PCa-specific homing and metastasis to the bone. Once PCa is disseminated in the bone, the cross talk between the bone microenvironmental factors and the PCa tumor cells contribute to the establishment of osteoblastic lesions. The factors mediating this cross talk and their signaling pathways need to be further delineated to ultimately halt the progression of metastatic lesions in the bone. The development of better animal models that fully recapitulate the metastatic process as seen in human disease is paramount to deciphering the molecular events associated with PCa metastasis.

AUTHOR CONTRIBUTIONS

Dr. Sourik S. Ganguly drafted, helped revise, and designed the figures for the manuscript. Dr. Xiaohong Li revised and provided intellectual content. Dr. Cindy K. Miranti revised, provided intellectual content, and finalized the manuscript.

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REFERENCES

1. Center MM, Jemal A, Lortet-Tieulent J, Ward E, Ferlay J, Brawley O, et al. International variation in prostate cancer incidence and mortality rates. *Eur Urol* (2012) **61**(6):1079–92. doi:10.1016/j.eururo.2012.02.054
2. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. *CA Cancer J Clin* (2013) **63**(1):11–30. doi:10.3322/caac.21166
3. Coleman RE. Clinical features of metastatic bone disease and risk of skeletal morbidity. *Clin Cancer Res* (2006) **12**(20 Pt 2):6243s–9s. doi:10.1158/1078-0432.CCR-06-0931
4. Hoimes CJ, Kelly WK. Redefining hormone resistance in prostate cancer. *Ther Adv Med Oncol* (2010) **2**(2):107–23. doi:10.1177/1758834009356433
5. Karantanos T, Corn PG, Thompson TC. Prostate cancer progression after androgen deprivation therapy: mechanisms of castrate resistance and novel therapeutic approaches. *Oncogene* (2013) **32**(49):5501–11. doi:10.1038/onc.2013.206
6. Linja MJ, Savinainen KJ, Saramaki OR, Tammela TL, Vessella RL, Visakorpi T. Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. *Cancer Res* (2001) **61**(9):3550–5.
7. Ruizeveld de Winter JA, Janssen PJ, Sleddens HM, Verleun-Mooijman MC, Trapman J, Brinkmann AO, et al. Androgen receptor status in localized and locally progressive hormone refractory human prostate cancer. *Am J Pathol* (1994) **144**(4):735–46.
8. van der Kwast TH, Schalken J, Ruizeveld de Winter JA, van Vroonhoven CC, Mulder E, Boersma W, et al. Androgen receptors in endocrine-therapy-resistant human prostate cancer. *Int J Cancer* (1991) **48**(2):189–93. doi:10.1002/ijc.2910480206
9. Stanbrough M, Bubley GJ, Ross K, Golub TR, Rubin MA, Penning TM, et al. Increased expression of genes converting adrenal androgens to testosterone in androgen-independent prostate cancer. *Cancer Res* (2006) **66**(5):2815–25. doi:10.1158/0008-5472.CAN-05-4000
10. Azzouni F, Godoy A, Li Y, Mohler J. The 5 α-reductase isozyme family: a review of basic biology and their role in human diseases. *Adv Urol* (2012) **2012**:530121. doi:10.1155/2012/530121
11. Zobniw CM, Causebrook A, Fong MK. Clinical use of abiraterone in the treatment of metastatic castration-resistant prostate cancer. *Res Rep Urol* (2014) **6**:97–105. doi:10.2147/RRU.S29003
12. Eisermann K, Wang D, Jing Y, Pascal LE, Wang Z. Androgen receptor gene mutation, rearrangement, polymorphism. *Transl Androl Urol* (2013) **2**(3):137–47. doi:10.3978/j.issn.2223-4683.2013.09.15
13. Li Y, Chan SC, Brand LJ, Hwang TH, Silverstein KA, Dehm SM. Androgen receptor splice variants mediate enzalutamide resistance in castration-resistant

prostate cancer cell lines. *Cancer Res* (2013) **73**(2):483–9. doi:10.1158/0008-5472.CAN-12-3630
14. Foradori CD, Weiser MJ, Handa RJ. Non-genomic actions of androgens. *Front Neuroendocrinol* (2008) **29**(2):169–81. doi:10.1016/j.yfrne.2007.10.005
15. Migliaccio A, Castoria G, Auricchio F. Analysis of androgen receptor rapid actions in cellular signaling pathways: receptor/Src association. *Methods Mol Biol* (2011) **776**:361–70. doi:10.1007/978-1-61779-243-4_21
16. Corn PG. The tumor microenvironment in prostate cancer: elucidating molecular pathways for therapy development. *Cancer Manag Res* (2012) **4**:183–93. doi:10.2147/CMAR.S32839
17. Alphonso A, Alahari SK. Stromal cells and Integrins: conforming to the needs of the tumor microenvironment. *Neoplasia* (2009) **11**(12):1264–71. doi:10.1593/neo.91302
18. Olumi AF, Grossfeld GD, Hayward SW, Carroll PR, Tlsty TD, Cunha GR. Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. *Cancer Res* (1999) **59**(19):5002–11.
19. Bhowmick NA, Neilson EG, Moses HL. Stromal fibroblasts in cancer initiation and progression. *Nature* (2004) **432**(7015):332–7. doi:10.1038/nature03096
20. Schauer IG, Sood AK, Mok S, Liu J. Cancer-associated fibroblasts and their putative role in potentiating the initiation and development of epithelial ovarian cancer. *Neoplasia* (2011) **13**(5):393–405. doi:10.1593/neo.101720
21. Tuxhorn JA, McAlhany SJ, Dang TD, Ayala GE, Rowley DR. Stromal cells promote angiogenesis and growth of human prostate tumors in a differential reactive stroma (DRS) xenograft model. *Cancer Res* (2002) **62**(11):3298–307.
22. Tuxhorn JA, Ayala GE, Rowley DR. Reactive stroma in prostate cancer progression. *J Urol* (2001) **166**(6):2472–83. doi:10.1097/00005392-200112000-00126
23. Horvath LG, Henshall SM, Lee CS, Kench JG, Golovsky D, Brenner PC, et al. Lower levels of nuclear β-catenin predict for a poorer prognosis in localized prostate cancer. *Int J Cancer* (2005) **113**(3):415–22. doi:10.1002/ijc.20599
24. Frank SB, Miranti CK. Disruption of prostate epithelial differentiation pathways and prostate cancer development. *Front Oncol* (2013) **3**:273. doi:10.3389/fonc.2013.00273
25. Schmelz M, Cress AE, Scott KM, Burger F, Cui H, Sallam K, et al. Different phenotypes in human prostate cancer: α6 or α3 integrin in cell-extracellular adhesion sites. *Neoplasia* (2002) **4**(3):243–54. doi:10.1038/sj.neo.7900223
26. Danen EH, van Muijen GN, van de Wiel-van Kemenade E, Jansen KF, Ruiter DJ, Figdor CG. Regulation of integrin-mediated adhesion to laminin and collagen in human melanocytes and in non-metastatic and highly metastatic human melanoma cells. *Int J Cancer* (1993) **54**(2):315–21. doi:10.1002/ijc.2910540225
27. Friedrichs K, Ruiz P, Franke F, Gille I, Terpe HJ, Imhof BA. High expression level of alpha 6 integrin in human breast carcinoma is correlated with reduced survival. *Cancer Res* (1995) **55**(4):901–6.
28. Ports MO, Nagle RB, Pond GD, Cress AE. Extracellular engagement of α6 integrin inhibited urokinase-type plasminogen activator-mediated cleavage and delayed human prostate bone metastasis. *Cancer Res* (2009) **69**(12):5007–14. doi:10.1158/0008-5472.CAN-09-0354
29. Lamb LE, Zarif JC, Miranti CK. The androgen receptor induces integrin α6β1 to promote prostate tumor cell survival via NF-κB and Bcl-xL Independently of PI3K signaling. *Cancer Res* (2011) **71**(7):2739–49. doi:10.1158/0008-5472.CAN-10-2745
30. Sroka IC, Anderson TA, McDaniel KM, Nagle RB, Gretzer MB, Cress AE. The laminin binding integrin α6β1 in prostate cancer perineural invasion. *J Cell Physiol* (2010) **224**(2):283–8. doi:10.1002/jcp.22149
31. Bonkhoff H, Stein U, Remberger K. Differential expression of α6 and α2 very late antigen integrins in the normal, hyperplastic, and neoplastic prostate: simultaneous demonstration of cell surface receptors and their extracellular ligands. *Hum Pathol* (1993) **24**(3):243–8. doi:10.1016/0046-8177(93)90033-D
32. Siler U, Rousselle P, Muller CA, Klein G. Laminin γ2 chain as a stromal cell marker of the human bone marrow microenvironment. *Br J Haematol* (2002) **119**(1):212–20. doi:10.1046/j.1365-2141.2002.03800.x
33. Siler U, Seiffert M, Puch S, Richards A, Torok-Storb B, Muller CA, et al. Characterization and functional analysis of laminin isoforms in human bone marrow. *Blood* (2000) **96**(13):4194–203.
34. Jin R, Yi Y, Yull FE, Blackwell TS, Clark PE, Koyama T, et al. NF-κB gene signature predicts prostate cancer progression. *Cancer Res* (2014) **74**(10):2763–72. doi:10.1158/0008-5472.CAN-13-2543
35. Lessard L, Begin LR, Gleave ME, Mes-Masson AM, Saad F. Nuclear localisation of nuclear factor-κB transcription factors in prostate cancer: an immunohistochemical study. *Br J Cancer* (2005) **93**(9):1019–23. doi:10.1038/sj.bjc.6602796
36. Jin RJ, Lho Y, Connelly L, Wang Y, Yu X, Saint Jean L, et al. The nuclear factor-kappaB pathway controls the progression of prostate cancer to androgen-independent growth. *Cancer Res* (2008) **68**(16):6762–9. doi:10.1158/0008-5472.CAN-08-0107
37. Fornaro M, Tallini G, Zheng DQ, Flanagan WM, Manzotti M, Languino LR. p27(kip1) acts as a downstream effector of and is coexpressed with the β1C integrin in prostatic adenocarcinoma. *J Clin Invest* (1999) **103**(3):321–9. doi:10.1172/JCI4585
38. Pontes-Junior J, Reis ST, Dall'Oglio M, Neves de Oliveira LC, Cury J, Carvalho PA, et al. Evaluation of the expression of integrins and cell adhesion molecules through tissue microarray in lymph node metastases of prostate cancer. *J Carcinog* (2009) **8**:3. doi:10.4103/1477-3163.48453
39. Demetriou MC, Pennington ME, Nagle RB, Cress AE. Extracellular α6 integrin cleavage by urokinase-type plasminogen activator in human prostate cancer. *Exp Cell Res* (2004) **294**(2):550–8. doi:10.1016/j.yexcr.2003.11.023
40. Pawar SC, Demetriou MC, Nagle RB, Bowden GT, Cress AE. Integrin alpha6 cleavage: a novel modification to modulate cell migration. *Exp Cell Res* (2007) **313**(6):1080–9. doi:10.1016/j.yexcr.2007.01.006
41. Landowski TH, Gard J, Pond E, Pond GD, Nagle RB, Geffre CP, et al. Targeting integrin α6 stimulates curative-type bone metastasis lesions in a xenograft model. *Mol Cancer Ther* (2014) **13**(6):1558–66. doi:10.1158/1535-7163.MCT-13-0962
42. Pawar SC, Dougherty S, Pennington ME, Demetriou MC, Stea BD, Dorr RT, et al. α6 integrin cleavage: sensitizing human prostate cancer to ionizing radiation. *Int J Radiat Biol* (2007) **83**(11–12):761–7. doi:10.1080/09553000701633135
43. King TE, Pawar SC, Majuta L, Sroka IC, Wynn D, Demetriou MC, et al. The role of α6 integrin in prostate cancer migration and bone pain in a novel xenograft model. *PLoS One* (2008) **3**(10):e3535. doi:10.1371/journal.pone.0003535
44. Miranti CK. Controlling cell surface dynamics and signaling: how CD82/KAI1 suppresses metastasis. *Cell Signal* (2009) **21**(2):196–211. doi:10.1016/j.cellsig.2008.08.023
45. Dong JT, Lamb PW, Rinker-Schaeffer CW, Vukanovic J, Ichikawa T, Isaacs JT, et al. KAI1, a metastasis suppressor gene for prostate cancer on human chromosome 11p11.2. *Science* (1995) **268**(5212):884–6. doi:10.1126/science.7754374
46. Dong JT, Suzuki H, Pin SS, Bova GS, Schalken JA, Isaacs WB, et al. Down-regulation of the KAI1 metastasis suppressor gene during the progression of human prostatic cancer infrequently involves gene mutation or allelic loss. *Cancer Res* (1996) **56**(19):4387–90.
47. He B, Liu L, Cook GA, Grgurevich S, Jennings LK, Zhang XA. Tetraspanin CD82 attenuates cellular morphogenesis through down-regulating integrin α6-mediated cell adhesion. *J Biol Chem* (2005) **280**(5):3346–54. doi:10.1074/jbc.M406680200
48. Sridhar SC, Miranti CK. Tetraspanin KAI1/CD82 suppresses invasion by inhibiting integrin-dependent crosstalk with c-Met receptor and Src kinases. *Oncogene* (2006) **25**(16):2367–78. doi:10.1038/sj.onc.1209269
49. Han SY, Lee M, Hong YK, Hwang S, Choi G, Suh YS, et al. Tsp66E, the Drosophila KAI1 homologue, and Tsp74F function to regulate ovarian follicle cell and wing development by stabilizing integrin localization. *FEBS Lett* (2012) **586**(22):4031–7. doi:10.1016/j.febslet.2012.09.044
50. Termini CM, Cotter ML, Marjon KD, Buranda T, Lidke KA, Gillette JM. The membrane scaffold CD82 regulates cell adhesion by altering α4 integrin stability and molecular density. *Mol Biol Cell* (2014) **25**(10):1560–73. doi:10.1091/mbc.E13-11-0660
51. Abe M, Sugiura T, Takahashi M, Ishii K, Shimoda M, Shirasuna K. A novel function of CD82/KAI-1 on E-cadherin-mediated homophilic cellular adhesion of cancer cells. *Cancer Lett* (2008) **266**(2):163–70. doi:10.1016/j.canlet.2008.02.058
52. Sottnik JL, Daignault-Newton S, Zhang X, Morrissey C, Hussain MH, Keller ET, et al. Integrin α2β1 (α2β1) promotes prostate cancer skeletal metastasis. *Clin Exp Metastasis* (2013) **30**(5):569–78. doi:10.1007/s10585-012-9561-6
53. Ziaee S, Chung LW. Induction of integrin α2 in a highly bone metastatic human prostate cancer cell line: roles of RANKL and AR under three-dimensional suspension culture. *Mol Cancer* (2014) **13**:208. doi:10.1186/1476-4598-13-208
54. Mason SD, Joyce JA. Proteolytic networks in cancer. *Trends Cell Biol* (2011) **21**(4):228–37. doi:10.1016/j.tcb.2010.12.002

55. Klezovitch O, Chevillet J, Mirosevich J, Roberts RL, Matusik RJ, Vasioukhin V. Hepsin promotes prostate cancer progression and metastasis. *Cancer Cell* (2004) **6**(2):185–95. doi:10.1016/j.ccr.2004.07.008
56. Jung H, Lee KP, Park SJ, Park JH, Jang YS, Choi SY, et al. TMPRSS4 promotes invasion, migration and metastasis of human tumor cells by facilitating an epithelial-mesenchymal transition. *Oncogene* (2008) **27**(18):2635–47. doi:10.1038/sj.onc.1210914
57. Tang X, Mahajan SS, Nguyen LT, Beliveau F, Leduc R, Simon JA, et al. Targeted inhibition of cell-surface serine protease Hepsin blocks prostate cancer bone metastasis. *Oncotarget* (2014) **5**(5):1352–62.
58. Holt SK, Kwon EM, Lin DW, Ostrander EA, Stanford JL. Association of hepsin gene variants with prostate cancer risk and prognosis. *Prostate* (2010) **70**(9):1012–9. doi:10.1002/pros.21135
59. Kim HJ, Han JH, Chang IH, Kim W, Myung SC. Variants in the HEPSIN gene are associated with susceptibility to prostate cancer. *Prostate Cancer Prostatic Dis* (2012) **15**(4):353–8. doi:10.1038/pcan.2012.17
60. Kirchhofer D, Peek M, Lipari MT, Billeci K, Fan B, Moran P. Hepsin activates pro-hepatocyte growth factor and is inhibited by hepatocyte growth factor activator inhibitor-1B (HAI-1B) and HAI-2. *FEBS Lett* (2005) **579**(9):1945–50. doi:10.1016/j.febslet.2005.01.085
61. Tripathi M, Nandana S, Yamashita H, Ganesan R, Kirchhofer D, Quaranta V. Laminin-332 is a substrate for hepsin, a protease associated with prostate cancer progression. *J Biol Chem* (2008) **283**(45):30576–84. doi:10.1074/jbc.M802312200
62. Dhanasekaran SM, Barrette TR, Ghosh D, Shah R, Varambally S, Kurachi K, et al. Delineation of prognostic biomarkers in prostate cancer. *Nature* (2001) **412**(6849):822–6. doi:10.1038/35090585
63. Wu SR, Cheng TS, Chen WC, Shyu HY, Ko CJ, Huang HP, et al. Matriptase is involved in ErbB-2-induced prostate cancer cell invasion. *Am J Pathol* (2010) **177**(6):3145–58. doi:10.2353/ajpath.2010.100228
64. Takeuchi T, Shuman MA, Craik CS. Reverse biochemistry: use of macromolecular protease inhibitors to dissect complex biological processes and identify a membrane-type serine protease in epithelial cancer and normal tissue. *Proc Natl Acad Sci U S A* (1999) **96**(20):11054–61. doi:10.1073/pnas.96.20.11054
65. Davis TL, Cress AE, Dalkin BL, Nagle RB. Unique expression pattern of the α6β4 integrin and laminin-5 in human prostate carcinoma. *Prostate* (2001) **46**(3):240–8. doi:10.1002/1097-0045(20010215)46:3<240::AID-PROS1029>3.3.CO;2-S
66. Kiyomiya K, Lee MS, Tseng IC, Zuo H, Barndt RJ, Johnson MD, et al. Matriptase activation and shedding with HAI-1 is induced by steroid sex hormones in human prostate cancer cells, but not in breast cancer cells. *Am J Physiol Cell Physiol* (2006) **291**(1):C40–9. doi:10.1152/ajpcell.00351.2005
67. Miller GS, List K. The matriptase-prostasin proteolytic cascade in epithelial development and pathology. *Cell Tissue Res* (2013) **351**(2):245–53. doi:10.1007/s00441-012-1348-1
68. Abate-Shen C, Shen MM. Molecular genetics of prostate cancer. *Genes Dev* (2000) **14**(19):2410–34. doi:10.1101/gad.819500
69. Chen YW, Lee MS, Lucht A, Chou FP, Huang W, Havighurst TC, et al. TMPRSS2, a serine protease expressed in the prostate on the apical surface of luminal epithelial cells and released into semen in prostasomes, is misregulated in prostate cancer cells. *Am J Pathol* (2010) **176**(6):2986–96. doi:10.2353/ajpath.2010.090665
70. Balk SP, Ko YJ, Bubley GJ. Biology of prostate-specific antigen. *J Clin Oncol* (2003) **21**(2):383–91. doi:10.1200/JCO.2003.02.083
71. Lucas JM, True L, Hawley S, Matsumura M, Morrissey C, Vessella R, et al. The androgen-regulated type II serine protease TMPRSS2 is differentially expressed and mislocalized in prostate adenocarcinoma. *J Pathol* (2008) **215**(2):118–25. doi:10.1002/path.2330
72. Lu P, Takai K, Weaver VM, Werb Z. Extracellular matrix degradation and remodeling in development and disease. *Cold Spring Harb Perspect Biol* (2011) **3**(12):1–24. doi:10.1101/cshperspect.a005058
73. Westermarck J, Kähäri VM. Regulation of matrix metalloproteinase expression in tumor invasion. *FASEB J* (1999) **13**(8):781–92.
74. Loo WT, Cheung MN, Chow LW. Production of matrix metalloproteinases in specific subpopulations of human-patient breast cancer invading in three dimensional cultural system. *Life Sci* (2004) **76**(7):743–52. doi:10.1016/j.lfs.2004.06.027
75. Nelson AR, Fingleton B, Rothenberg ML, Matrisian LM. Matrix metalloproteinases: biologic activity and clinical implications. *J Clin Oncol* (2000) **18**(5):1135–49. doi:10.1016/j.lfs.2004.06.027
76. Benaud C, Dickson RB, Thompson EW. Matrix metalloproteinases in mammary gland development and cancer. *Breast Cancer Res Treat* (1998) **50**(2):97–116. doi:10.1023/A:1006061115909
77. Ganguly SS, Fiore LS, Sims JT, Friend JW, Srinivasan D, Thacker MA, et al. c-Abl and Arg are activated in human primary melanomas, promote melanoma cell invasion via distinct pathways, and drive metastatic progression. *Oncogene* (2012) **31**(14):1804–16. doi:10.1038/onc.2011.361
78. Morgia G, Falsaperla M, Malaponte G, Madonia M, Indelicato M, Travali S, et al. Matrix metalloproteinases as diagnostic (MMP-13) and prognostic (MMP-2, MMP-9) markers of prostate cancer. *Urol Res* (2005) **33**(1):44–50. doi:10.1007/s00240-004-0440-8
79. Wood M, Fudge K, Mohler JL, Frost AR, Garcia F, Wang M, et al. In situ hybridization studies of metalloproteinases 2 and 9 and TIMP-1 and TIMP-2 expression of human prostate cancer. *Clin Cancer Res* (1997) **15**(3):246–58.
80. Incorvaia L, Badalamenti G, Rini G, Arcara C, Fricano S, Sferrazza C, et al. MMP-2, MMP-9 and activin A blood levels in patients with breast cancer or prostate cancer metastatic to the bone. *Anticancer Res* (2007) **27**(3B):1519–25.
81. Pulukuri SM, Rao JS. Matrix metalloproteinase-1 promotes prostate tumor growth and metastasis. *Int J Oncol* (2008) **32**(4):757–65. doi:10.3892/ijo.32.4.757
82. Xu D, McKee CM, Cao Y, Ding Y, Kessler BM, Muschel RJ. Matrix metalloproteinase-9 regulates tumor cell invasion through cleavage of protease nexin-1. *Cancer Res* (2010) **70**(17):6988–98. doi:10.1158/0008-5472.CAN-10-0242
83. Pulukuri SM, Gondi CS, Lakka SS, Jutla A, Estes N, Gujrati M, et al. RNA interference-directed knockdown of urokinase plasminogen activator and urokinase plasminogen activator receptor inhibits prostate cancer cell invasion, survival, and tumorigenicity *in vivo*. *J Biol Chem* (2005) **280**(43):36529–40. doi:10.1074/jbc.M503111200
84. Jin JK, Dayyani F, Gallick GE. Steps in prostate cancer progression that lead to bone metastasis. *Int J Cancer* (2011) **128**(11):2545–61. doi:10.1002/ijc.26024
85. Coussens LM, Fingleton B, Matrisian LM. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. *Science* (2002) **295**(5564):2387–92. doi:10.1126/science.1067100
86. Cheng TS, Chen WC, Lin YY, Tsai CH, Liao CI, Shyu HY, et al. Curcumin-targeting pericellular serine protease matriptase role in suppression of prostate cancer cell invasion, tumor growth, and metastasis. *Cancer Prev Res (Phila)* (2013) **6**(5):495–505. doi:10.1158/1940-6207.CAPR-12-0293-T
87. Ganesan R, Eigenbrot C, Kirchhofer D. Structural and mechanistic insight into how antibodies inhibit serine proteases. *Biochem J* (2010) **430**(2):179–89. doi:10.1042/BJ20100634
88. Ihara S, Miyoshi E, Ko JH, Murata K, Nakahara S, Honke K, et al. Prometastatic effect of N-acetylglucosaminyltransferase V is due to modification and stabilization of active matriptase by adding beta 1-6 GlcNAc branching. *J Biol Chem* (2002) **277**(19):16960–7. doi:10.1074/jbc.M200673200
89. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* (2011) **144**(5):646–74. doi:10.1016/j.cell.2011.02.013
90. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol* (2014) **15**(3):178–96. doi:10.1038/nrm3758
91. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* (2008) **133**(4):704–15. doi:10.1016/j.cell.2008.03.027
92. Rybinski B, Franco-Barraza J, Cukierman E. The wound healing, chronic fibrosis, and cancer progression triad. *Physiol Genomics* (2014) **46**(7):223–44. doi:10.1152/physiolgenomics.00158.2013
93. Adams CL, Chen YT, Smith SJ, Nelson WJ. Mechanisms of epithelial cell-cell adhesion and cell compaction revealed by high-resolution tracking of E-cadherin-green fluorescent protein. *J Cell Biol* (1998) **142**(4):1105–19. doi:10.1083/jcb.142.4.1105
94. Umbas R, Isaacs WB, Bringuier PP, Schaafsma HE, Karthaus HF, Oosterhof GO, et al. Decreased E-cadherin expression is associated with poor prognosis in patients with prostate cancer. *Cancer Res* (1994) **54**(14):3929–33.

95. Shen MM, Abate-Shen C. Molecular genetics of prostate cancer: new prospects for old challenges. *Genes Dev* (2010) **24**(18):1967–2000. doi:10.1101/gad.1965810
96. Chaudhry P, Fabi F, Singh M, Parent S, Leblanc V, Asselin E. Prostate apoptosis response-4 mediates TGF-β-induced epithelial-to-mesenchymal transition. *Cell Death Dis* (2014) **5**:e1044. doi:10.1038/cddis.2014.7
97. Derynck R, Akhurst RJ, Balmain A. TGF-β signaling in tumor suppression and cancer progression. *Nat Genet* (2001) **29**(2):117–29. doi:10.1038/ng1001-117
98. Garg M. Epithelial-mesenchymal transition – activating transcription factors – multifunctional regulators in cancer. *World J Stem Cells* (2013) **5**(4):188–95. doi:10.4252/wjsc.v5.i4.188
99. Vincent T, Neve EP, Johnson JR, Kukalev A, Rojo F, Albanell J, et al. A SNAIL1-SMAD3/4 transcriptional repressor complex promotes TGF-β mediated epithelial-mesenchymal transition. *Nat Cell Biol* (2009) **11**(8):943–50. doi:10.1038/ncb1905
100. Zavadil J, Bottinger EP. TGF-β and epithelial-to-mesenchymal transitions. *Oncogene* (2005) **24**(37):5764–74. doi:10.1038/sj.onc.1208927
101. Puisieux A, Brabletz T, Caramel J. Oncogenic roles of EMT-inducing transcription factors. *Nat Cell Biol* (2014) **16**(6):488–94. doi:10.1038/ncb2976
102. Putzke AP, Ventura AP, Bailey AM, Akture C, Opoku-Ansah J, Celiktas M, et al. Metastatic progression of prostate cancer and e-cadherin regulation by zeb1 and SRC family kinases. *Am J Pathol* (2011) **179**(1):400–10. doi:10.1016/j.ajpath.2011.03.028
103. Jacob S, Nayak S, Fernandes G, Barai RS, Menon S, Chaudhari UK, et al. Androgen receptor as a regulator of ZEB2 expression and its implications in epithelial-to-mesenchymal transition in prostate cancer. *Endocr Relat Cancer* (2014) **21**(3):473–86. doi:10.1530/ERC-13-0514
104. He H, Yang X, Davidson AJ, Wu D, Marshall FF, Chung LW, et al. Progressive epithelial to mesenchymal transitions in ARCaP E prostate cancer cells during xenograft tumor formation and metastasis. *Prostate* (2010) **70**(5):518–28. doi:10.1002/pros.21086
105. Etzioni R, Cha R, Feuer EJ, Davidov O. Asymptomatic incidence and duration of prostate cancer. *Am J Epidemiol* (1998) **148**(8):775–85. doi:10.1093/oxfordjournals.aje.a009698
106. Tsai JH, Donaher JL, Murphy DA, Chau S, Yang J. Spatiotemporal regulation of epithelial-mesenchymal transition is essential for squamous cell carcinoma metastasis. *Cancer Cell* (2012) **22**(6):725–36. doi:10.1016/j.ccr.2012.09.022
107. Labelle M, Begum S, Hynes RO. Direct signaling between platelets and cancer cells induces an epithelial-mesenchymal-like transition and promotes metastasis. *Cancer Cell* (2011) **20**(5):576–90. doi:10.1016/j.ccr.2011.09.009
108. Vral A, Magri V, Montanari E, Gazzano G, Gourvas V, Marras E, et al. Topographic and quantitative relationship between prostate inflammation, proliferative inflammatory atrophy and low-grade prostate intraepithelial neoplasia: a biopsy study in chronic prostatitis patients. *Int J Oncol* (2012) **41**(6):1950–8. doi:10.3892/ijo.2012.1646
109. Kwon OJ, Zhang L, Ittmann MM, Xin L. Prostatic inflammation enhances basal-to-luminal differentiation and accelerates initiation of prostate cancer with a basal cell origin. *Proc Natl Acad Sci U S A* (2014) **111**(5):E592–600. doi:10.1073/pnas.1318157111
110. Valencia T, Kim JY, Abu-Baker S, Moscat-Pardos J, Ahn CS, Reina-Campos M, et al. Metabolic reprogramming of stromal fibroblasts through p62-mTORC1 signaling promotes inflammation and tumorigenesis. *Cancer Cell* (2014) **26**(1):121–35. doi:10.1016/j.ccr.2014.05.004
111. Brennen WN, Denmeade SR, Isaacs JT. Mesenchymal stem cells as a vector for the inflammatory prostate microenvironment. *Endocr Relat Cancer* (2013) **20**(5):R269–90. doi:10.1530/ERC-13-0151
112. Herroon MK, Rajagurubandara E, Hardaway AL, Powell K, Turchick A, Feldmann D, et al. Bone marrow adipocytes promote tumor growth in bone via FABP4-dependent mechanisms. *Oncotarget* (2013) **4**(11):2108–23.
113. Nguyen DP, Li J, Yadav SS, Tewari AK. Recent insights into NF-κB signalling pathways and the link between inflammation and prostate cancer. *BJU Int* (2014) **114**(2):168–76. doi:10.1111/bju.12488
114. Charles KA, Kulbe H, Soper R, Escorcio-Correia M, Lawrence T, Schultheis A, et al. The tumor-promoting actions of TNF-α involve TNFR1 and IL-17 in ovarian cancer in mice and humans. *J Clin Invest* (2009) **119**(10):3011–23. doi:10.1172/JCI39065
115. Sullivan DE, Ferris M, Nguyen H, Abboud E, Brody AR. TNF-α induces TGF-β1expression in lung fibroblasts at the transcriptional levelviaAP-1 activation. *J Cell Mol Med* (2009) **13**(8b):1866–76. doi:10.1111/j.1582-4934.2009.00647.x
116. Bates RC, Mercurio AM. Tumor necrosis factor-α stimulates the epithelial-to-mesenchymal transition of human colonic organoids. *Mol Biol Cell* (2003) **14**(5):1790–800. doi:10.1091/mbc.E02-09-0583
117. Kokudo T, Suzuki Y, Yoshimatsu Y, Yamazaki T, Watabe T, Miyazono K. Snail is required for TGFbeta-induced endothelial-mesenchymal transition of embryonic stem cell-derived endothelial cells. *J Cell Sci* (2008) **121**(Pt 20):3317–24. doi:10.1242/jcs.028282
118. Naber HP, Drabsch Y, Snaar-Jagalska BE, ten Dijke P, van Laar T. Snail and Slug, key regulators of TGF-β-induced EMT, are sufficient for the induction of single-cell invasion. *Biochem Biophys Res Commun* (2013) **435**(1):58–63. doi:10.1016/j.bbrc.2013.04.037
119. Wang H, Wang HS, Zhou BH, Li CL, Zhang F, Wang XF, et al. Epithelial-mesenchymal transition (EMT) induced by TNF-α requires AKT/GSK-3beta-mediated stabilization of snail in colorectal cancer. *PLoS One* (2013) **8**(2):e56664. doi:10.1371/journal.pone.0056664
120. Li X, Xu Y, Chen Y, Chen S, Jia X, Sun T, et al. SOX2 promotes tumor metastasis by stimulating epithelial-to-mesenchymal transition via regulation of WNT/beta-catenin signal network. *Cancer Lett* (2013) **336**(2):379–89. doi:10.1016/j.canlet.2013.03.027
121. Furusato B, Tan SH, Young D, Dobi A, Sun C, Mohamed AA, et al. ERG oncoprotein expression in prostate cancer: clonal progression of ERG-positive tumor cells and potential for ERG-based stratification. *Prostate Cancer Prostatic Dis* (2010) **13**(3):228–37. doi:10.1038/pcan.2010.23
122. Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW, et al. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. *Science* (2005) **310**(5748):644–8. doi:10.1126/science.1117679
123. Gupta S, Iljin K, Sara H, Mpindi JP, Mirtti T, Vainio P, et al. FZD4 as a mediator of ERG oncogene-induced WNT signaling and epithelial-to-mesenchymal transition in human prostate cancer cells. *Cancer Res* (2010) **70**(17):6735–45. doi:10.1158/0008-5472.CAN-10-0244
124. Michieli P. Hypoxia, angiogenesis and cancer therapy: to breathe or not to breathe? *Cell Cycle* (2009) **8**(20):3291–6. doi:10.4161/cc.8.20.9741
125. Pennacchietti S, Michieli P, Galluzzo M, Mazzone M, Giordano S, Comoglio PM. Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. *Cancer Cell* (2003) **3**(4):347–61. doi:10.1016/S1535-6108(03)00085-0
126. Zhao JH, Luo Y, Jiang YG, He DL, Wu CT. Knockdown of β-Catenin through shRNA cause a reversal of EMT and metastatic phenotypes induced by HIF-1α. *Cancer Invest* (2011) **29**(6):377–82. doi:10.3109/07357907.2010.512595
127. Boyce BF, Yoneda T, Guise TA. Factors regulating the growth of metastatic cancer in bone. *Endocr Relat Cancer* (1999) **6**(3):333–47. doi:10.1677/erc.0.0060333
128. Giunciuglio D, Cai T, Filanti C, Manduca P, Albini A. Effect of osteoblast supernatants on cancer cell migration and invasion. *Cancer Lett* (1995) **97**(1):69–74. doi:10.1016/0304-3835(95)03955-V
129. Jacob K, Webber M, Benayahu D, Kleinman HK. Osteonectin promotes prostate cancer cell migration and invasion: a possible mechanism for metastasis to bone. *Cancer Res* (1999) **59**:4453.
130. Festuccia C, Bologna M, Gravina GL, Guerra F, Angelucci A, Villanova I, et al. Osteoblast conditioned media contain TGF-beta1 and modulate the migration of prostate tumor cells and their interactions with extracellular matrix components. *Int J Cancer* (1999) **81**(3):395–403. doi:10.1002/(SICI)1097-0215(19990505)81:3<395::AID-IJC13>3.3.CO;2-M
131. Teicher BA, Fricker SP. CXCL12 (SDF-1)/CXCR4 pathway in cancer. *Clin Cancer Res* (2010) **16**(11):2927–31. doi:10.1158/1078-0432.CCR-09-2329
132. Salazar N, Castellan M, Shirodkar SS, Lokeshwar BL. Chemokines and chemokine receptors as promoters of prostate cancer growth and progression. *Crit Rev Eukaryot Gene Expr* (2013) **23**(1):77–91. doi:10.1615/CritRevEukaryotGeneExpr.2013006905
133. Das Roy L, Pathangey LB, Tinder TL, Schettini JL, Gruber HE, Mukherjee P. Breast-cancer-associated metastasis is significantly increased in a model of autoimmune arthritis. *Breast Cancer Res* (2009) **11**(4):R56. doi:10.1186/bcr2345
134. Uygur B, Wu WS. SLUG promotes prostate cancer cell migration and invasion via CXCR4/CXCL12 axis. *Mol Cancer* (2011) **10**:139. doi:10.1186/1476-4598-10-139

135. Sun YX, Schneider A, Jung Y, Wang J, Dai J, Wang J, et al. Skeletal localization and neutralization of the SDF-1(CXCL12)/CXCR4 axis blocks prostate cancer metastasis and growth in osseous sites *in vivo*. *J Bone Miner Res* (2005) **20**(2):318–29. doi:10.1359/JBMR.041109
136. Taichman RS, Cooper C, Keller ET, Pienta KJ, Taichman NS, McCauley LK. Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. *Cancer Res* (2002) **16**(6):1832–7.
137. Conley-LaComb MK, Saliganan A, Kandagatla P, Chen YQ, Cher ML, Chinni SR. PTEN loss mediated Akt activation promotes prostate tumor growth and metastasis via CXCL12/CXCR4 signaling. *Mol Cancer* (2013) **12**(1):85. doi:10.1186/1476-4598-12-85
138. Shiozawa Y, Pienta KJ, Taichman RS. Hematopoietic stem cell niche is a potential therapeutic target for bone metastatic tumors. *Clin Cancer Res* (2011) **17**(17):5553–8. doi:10.1158/1078-0432.CCR-10-2505
139. Psaila B, Lyden D. The metastatic niche: adapting the foreign soil. *Nat Rev Cancer* (2009) **9**(4):285–93. doi:10.1038/nrc2621
140. Langley RR, Fidler IJ. The seed and soil hypothesis revisited – the role of tumor-stroma interactions in metastasis to different organs. *Int J Cancer* (2011) **128**(11):2527–35. doi:10.1002/ijc.26031
141. Kaplan RN, Riba RD, Zacharoulis S, Bramley AH, Vincent L, Costa C, et al. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature* (2005) **438**(7069):820–7. doi:10.1038/nature04186
142. Hiratsuka S, Watanabe A, Aburatani H, Maru Y. Tumour-mediated upregulation of chemoattractants and recruitment of myeloid cells predetermines lung metastasis. *Nat Cell Biol* (2006) **8**(12):1369–75. doi:10.1038/ncb1507
143. Deng J, Liu Y, Lee H, Herrmann A, Zhang W, Zhang C, et al. S1PR1-STAT3 signaling is crucial for myeloid cell colonization at future metastatic sites. *Cancer Cell* (2012) **21**(5):642–54. doi:10.1016/j.ccr.2012.03.039
144. Zhau HE, Li CL, Chung LW. Establishment of human prostate carcinoma skeletal metastasis models. *Cancer* (2000) **88**(12 Suppl):2995–3001. doi:10.1002/1097-0142(20000615)88:12+<2995::AID-CNCR15>3.3.CO;2-P
145. Amir E, Tannock IF. Prostate cancer: androgen deprivation therapy and bone loss. *Nat Rev Urol* (2009) **6**(12):642–4. doi:10.1038/nrurol.2009.218
146. Weinstein RS, Jilka RL, Parfitt AM, Manolagas SC. The effects of androgen deficiency on murine bone remodeling and bone mineral density are mediated via cells of the osteoblastic lineage. *Endocrinology* (1997) **138**(9):4013–21. doi:10.1210/endo.138.9.5359
147. Economos C, Morrissey C, Vessella RL. Circulating tumor cells as a marker of response: implications for determining treatment efficacy and evaluating new agents. *Curr Opin Urol* (2012) **22**(3):190–6. doi:10.1097/MOU.0b013e3283519b58
148. Koop S, MacDonald IC, Luzzi K, Schmidt EE, Morris VL, Grattan M, et al. Fate of melanoma cells entering the microcirculation: over 80% survive and extravasate. *Cancer Res* (1995) **55**(12):2520–3.
149. Wikman H, Vessella R, Pantel K. Cancer micrometastasis and tumour dormancy. *APMIS* (2008) **116**(7–8):754–70. doi:10.1111/j.1600-0463.2008.01033.x
150. Okazaki M, Takeshita S, Kawai S, Kikuno R, Tsujimura A, Kudo A, et al. Molecular cloning and characterization of OB-cadherin, a new member of cadherin family expressed in osteoblasts. *J Biol Chem* (1994) **269**(16):12092–8.
151. Kawaguchi J, Kii I, Sugiyama Y, Takeshita S, Kudo A. The transition of cadherin expression in osteoblast differentiation from mesenchymal cells: consistent expression of cadherin-11 in osteoblast lineage. *J Bone Miner Res* (2001) **16**(2):260–9. doi:10.1359/jbmr.2001.16.2.260
152. Chu K, Cheng CJ, Ye X, Lee YC, Zurita AJ, Chen DT, et al. Cadherin-11 promotes the metastasis of prostate cancer cells to bone. *Mol Cancer Res* (2008) **6**(8):1259–67. doi:10.1158/1541-7786.MCR-08-0077
153. Huang CF, Lira C, Chu K, Bilen MA, Lee YC, Ye X, et al. Cadherin-11 increases migration and invasion of prostate cancer cells and enhances their interaction with osteoblasts. *Cancer Res* (2010) **70**(11):4580–9. doi:10.1158/0008-5472.CAN-09-3016
154. Tamura D, Hiraga T, Myoui A, Yoshikawa H, Yoneda T. Cadherin-11 mediated interactions with bone marrow stromal/osteoblastic cells support selective colonization of breast cancer cells in bone. *Int J Oncol* (2008) **33**(1):17–24.
155. Logothetis CJ, Lin SH. Osteoblasts in prostate cancer metastasis to bone. *Nat Rev Cancer* (2005) **5**(1):21–8. doi:10.1038/nrc1528
156. Mishra A, Shiozawa Y, Pienta KJ, Taichman RS. Homing of cancer cells to the bone. *Cancer Microenviron* (2011) **4**(3):221–35. doi:10.1007/s12307-011-0083-6
157. Yin JJ, Pollock CB, Kelly K. Mechanisms of cancer metastasis to the bone. *Cell Res* (2005) **15**(1):57–62. doi:10.1038/sj.cr.7290266
158. Keller ET, Brown J. Prostate cancer bone metastases promote both osteolytic and osteoblastic activity. *J Cell Biochem* (2004) **91**(4):718–29. doi:10.1002/jcb.10662
159. Darby S, Cross SS, Brown NJ, Hamdy FC, Robson CN. BMP-6 over-expression in prostate cancer is associated with increased Id-1 protein and a more invasive phenotype. *J Pathol* (2008) **214**(3):394–404. doi:10.1002/path.2292
160. Katsuno Y, Hanyu A, Kanda H, Ishikawa Y, Akiyama F, Iwase T, et al. Bone morphogenetic protein signaling enhances invasion and bone metastasis of breast cancer cells through Smad pathway. *Oncogene* (2008) **27**(49):6322–33. doi:10.1038/onc.2008.232
161. Dai J, Keller J, Zhang J, Lu Y, Yao Z, Keller ET. Bone morphogenetic protein-6 promotes osteoblastic prostate cancer bone metastases through a dual mechanism. *Cancer Res* (2005) **65**(18):8274–85. doi:10.1158/0008-5472.CAN-05-1891
162. Bentley H, Hamdy FC, Hart KA, Seid JM, Williams JL, Johnstone D, et al. Expression of bone morphogenetic proteins in human prostatic adenocarcinoma and benign prostatic hyperplasia. *Br J Cancer* (1992) **66**(6):1159–63. doi:10.1038/bjc.1992.427
163. Raida M, Clement JH, Ameri K, Han C, Leek RD, Harris AL. Expression of bone morphogenetic protein 2 in breast cancer cells inhibits hypoxic cell death. *Int J Oncol* (2005) **26**(6):1465–70.
164. Clement JH, Raida M, Sanger J, Bicknell R, Liu J, Naumann A, et al. Bone morphogenetic protein 2 (BMP-2) induces *in vitro* invasion and *in vivo* hormone independent growth of breast carcinoma cells. *Int J Oncol* (2005) **27**(2):401–7. doi:10.3892/ijo.27.2.401
165. Feeley BT, Gamradt SC, Hsu WK, Liu N, Krenek L, Robbins P, et al. Influence of BMPs on the formation of osteoblastic lesions in metastatic prostate cancer. *J Bone Miner Res* (2005) **20**(12):2189–99. doi:10.1359/JBMR.050802
166. Abe E, Yamamoto M, Taguchi Y, Lecka-Czernik B, O'Brien CA, Economides AN, et al. Essential requirement of BMPs-2/4 for both osteoblast and osteoclast formation in murine bone marrow cultures from adult mice: antagonism by noggin. *J Bone Miner Res* (2000) **15**(4):663–73. doi:10.1359/jbmr.2000.15.4.663
167. Gori F, Thomas T, Hicok KC, Spelsberg TC, Riggs BL. Differentiation of human marrow stromal precursor cells: bone morphogenetic protein-2 increases OSF2/CBFA1, enhances osteoblast commitment, and inhibits late adipocyte maturation. *J Bone Miner Res* (1999) **14**(9):1522–35. doi:10.1359/jbmr.1999.14.9.1522
168. Jena N, Martin-Seisdedos C, McCue P, Croce CM. BMP7 null mutation in mice: developmental defects in skeleton, kidney, and eye. *Exp Cell Res* (1997) **230**(1):28–37. doi:10.1006/excr.1996.3411
169. Nishimura R, Hata K, Matsubara T, Wakabayashi M, Yoneda T. Regulation of bone and cartilage development by network between BMP signalling and transcription factors. *J Biochem* (2012) **151**(3):247–54. doi:10.1093/jb/mvs004
170. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, et al. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* (2002) **108**(1):17–29. doi:10.1016/S0092-8674(01)00622-5
171. Morrissey C, Brown LG, Pitts TE, Vessella RL, Corey E. Bone morphogenetic protein 7 is expressed in prostate cancer metastases and its effects on prostate tumor cells depend on cell phenotype and the tumor microenvironment. *Neoplasia* (2010) **12**(2):192–205.
172. Kobayashi A, Okuda H, Xing F, Pandey PR, Watabe M, Hirota S, et al. Bone morphogenetic protein 7 in dormancy and metastasis of prostate cancer stem-like cells in bone. *J Exp Med* (2011) **208**(13):2641–55. doi:10.1084/jem.20110840
173. Lee GT, Kang DI, Ha YS, Jung YS, Chung J, Min K, et al. Prostate cancer bone metastases acquire resistance to androgen deprivation via WNT5A-mediated BMP-6 induction. *Br J Cancer* (2014) **110**(6):1634–44. doi:10.1038/bjc.2014.23
174. Dai J, Hall CL, Escara-Wilke J, Mizokami A, Keller JM, Keller ET. Prostate cancer induces bone metastasis through Wnt-induced bone morphogenetic protein-dependent and independent mechanisms. *Cancer Res* (2008) **68**(14):5785–94. doi:10.1158/0008-5472.CAN-07-6541
175. Godebu E, Muldong M, Strasner A, Wu C, Park S, Woo JR, et al. PCSD1, a new patient-derived model of bone metastatic prostate cancer, is castrate-resistant in the bone-niche. *J Transl Med* (2014) **12**(1):275. doi:10.1186/s12967-014-0275-1

176. Nishimori H, Ehata S, Suzuki HI, Katsuno Y, Miyazono K. Prostate cancer cells and bone stromal cells mutually interact with each other through bone morphogenetic protein-mediated signals. *J Biol Chem* (2012) **287**(24):20037–46. doi:10.1074/jbc.M112.353094
177. Zunich SM, Douglas T, Valdovinos M, Chang T, Bushman W, Walterhouse D, et al. Paracrine sonic hedgehog signalling by prostate cancer cells induces osteoblast differentiation. *Mol Cancer* (2009) **8**:12. doi:10.1186/1476-4598-8-12
178. Zunich SM, Valdovinos M, Douglas T, Walterhouse D, Iannaccone P, Lamm ML. Osteoblast-secreted collagen upregulates paracrine Sonic hedgehog signaling by prostate cancer cells and enhances osteoblast differentiation. *Mol Cancer* (2012) **11**:30. doi:10.1186/1476-4598-11-30
179. Rosano L, Spinella F, Bagnato A. Endothelin 1 in cancer: biological implications and therapeutic opportunities. *Nat Rev Cancer* (2013) **13**(9):637–51. doi:10.1038/nrc3546
180. Nelson JB, Udan MS, Guruli G, Pflug BR. Endothelin-1 inhibits apoptosis in prostate cancer. *Neoplasia* (2005) **7**(7):631–7. doi:10.1593/neo.04787
181. Rosano L, Varmi M, Salani D, Di Castro V, Spinella F, Natali PG, et al. Endothelin-1 induces tumor proteinase activation and invasiveness of ovarian carcinoma cells. *Cancer Res* (2001) **61**(22):8340–6.
182. Kurihara Y, Kurihara H, Suzuki H, Kodama T, Maemura K, Nagai R, et al. Elevated blood pressure and craniofacial abnormalities in mice deficient in endothelin-1. *Nature* (1994) **368**(6473):703–10. doi:10.1038/368703a0
183. Nelson JB, Hedican SP, George DJ, Reddi AH, Piantadosi S, Eisenberger MA, et al. Identification of endothelin-1 in the pathophysiology of metastatic adenocarcinoma of the prostate. *Nat Med* (1995) **1**(9):944–9. doi:10.1038/nm0995-944
184. Yin JJ, Mohammad KS, Kakonen SM, Harris S, Wu-Wong JR, Wessale JL, et al. A causal role for endothelin-1 in the pathogenesis of osteoblastic bone metastases. *Proc Natl Acad Sci U S A* (2003) **100**(19):10954–9. doi:10.1073/pnas.1830978100
185. Rahim F, Hajizamani S, Mortaz E, Ahmadzadeh A, Shahjahani M, Shahrabi S, et al. Molecular regulation of bone marrow metastasis in prostate and breast cancer. *Bone Marrow Res* (2014) **2014**:405920. doi:10.1155/2014/405920
186. Chiao JW, Moonga BS, Yang YM, Kancherla R, Mittelman A, Wu-Wong JR, et al. Endothelin-1 from prostate cancer cells is enhanced by bone contact which blocks osteoclastic bone resorption. *Br J Cancer* (2000) **83**(3):360–5. doi:10.1054/bjoc.2000.1261
187. Nelson JB, Nabulsi AA, Vogelzang NJ, Breul J, Zonnenberg BA, Daliani DD, et al. Suppression of prostate cancer induced bone remodeling by the endothelin receptor A antagonist atrasentan. *J Urol* (2003) **169**(3):1143–9. doi:10.1097/01.ju.0000042162.08938.27
188. Quinn DI, Tangen CM, Hussain M, Lara PN, Goldkorn A, Moinpour CM, et al. Docetaxel and atrasentan versus docetaxel and placebo for men with advanced castration-resistant prostate cancer (SWOG S0421): a randomised phase 3 trial. *Lancet Oncol* (2013) **14**(9):893–900. doi:10.1016/S1470-2045(13)70294-8
189. Clines GA, Mohammad KS, Bao Y, Stephens OW, Suva LJ, Shaughnessy JD Jr., et al. Dickkopf homolog 1 mediates endothelin-1-stimulated new bone formation. *Mol Endocrinol* (2007) **21**(2):486–98. doi:10.1210/me.2006-0346
190. Anastas JN, Moon RT. WNT signalling pathways as therapeutic targets in cancer. *Nat Rev Cancer* (2013) **13**(1):11–26. doi:10.1038/nrc3419
191. Kypta RM, Waxman J. Wnt/beta-catenin signalling in prostate cancer. *Nat Rev Urol* (2012) **9**(8):418–28. doi:10.1038/nrurol.2012.116
192. Hall CL, Kang S, MacDougald OA, Keller ET. Role of Wnts in prostate cancer bone metastases. *J Cell Biochem* (2006) **97**(4):661–72. doi:10.1002/jcb.20735
193. Yardy GW, Brewster SF. Wnt signalling and prostate cancer. *Prostate Cancer Prostatic Dis* (2005) **8**(2):119–26. doi:10.1038/sj.pcan.4500794
194. Watanabe M, Kakiuchi H, Kato H, Shiraishi T, Yatani R, Sugimura T, et al. APC gene mutations in human prostate cancer. *Jpn J Clin Oncol* (1996) **26**(2):77–81. doi:10.1093/oxfordjournals.jjco.a023188
195. Majid S, Saini S, Dahiya R. Wnt signaling pathways in urological cancers: past decades and still growing. *Mol Cancer* (2012) **11**:7. doi:10.1186/1476-4598-11-7
196. Chen G, Shukeir N, Potti A, Sircar K, Aprikian A, Goltzman D, et al. Up-regulation of Wnt-1 and β-catenin production in patients with advanced metastatic prostate carcinoma: potential pathogenetic and prognostic implications. *Cancer* (2004) **101**(6):1345–56. doi:10.1002/cncr.20518
197. Boissan M, De Wever O, Lizarraga F, Wendum D, Poincloux R, Chignard N, et al. Implication of metastasis suppressor NM23-H1 in maintaining adherens junctions and limiting the invasive potential of human cancer cells. *Cancer Res* (2010) **70**(19):7710–22. doi:10.1158/0008-5472.CAN-10-1887
198. Gaur T, Lengner CJ, Hovhannisyan H, Bhat RA, Bodine PV, Komm BS, et al. Canonical WNT signaling promotes osteogenesis by directly stimulating Runx2 gene expression. *J Biol Chem* (2005) **280**(39):33132–40. doi:10.1074/jbc.M500608200
199. Cho YD, Yoon WJ, Kim WJ, Woo KM, Baek JH, Lee G, et al. Epigenetic modifications and canonical wingless/int-1 Class (WNT) signaling enable trans-differentiation of nonosteogenic cells into osteoblasts. *J Biol Chem* (2014) **289**(29):20120–8. doi:10.1074/jbc.M114.558064
200. Hall CL, Bafico A, Dai J, Aaronson SA, Keller ET. Prostate cancer cells promote osteoblastic bone metastases through Wnts. *Cancer Res* (2005) **65**(17):7554–60. doi:10.1158/0008-5472.CAN-05-1317
201. Heldin CH. Targeting the PDGF signaling pathway in tumor treatment. *Cell Commun Signal* (2013) **11**:97. doi:10.1186/1478-811X-11-97
202. Ustach CV, Huang W, Conley-LaComb MK, Lin CY, Che M, Abrams J, et al. A novel signaling axis of matriptase/PDGF-D/ss-PDGFR in human prostate cancer. *Cancer Res* (2010) **70**(23):9631–40. doi:10.1158/0008-5472.CAN-10-0511
203. Ustach CV, Taube ME, Hurst NJ Jr., Bhagat S, Bonfil RD, Cher ML, et al. A potential oncogenic activity of platelet-derived growth factor d in prostate cancer progression. *Cancer Res* (2004) **64**(5):1722–9. doi:10.1158/0008-5472.CAN-03-3047
204. Caplan AI, Correa D. PDGF in bone formation and regeneration: new insights into a novel mechanism involving MSCs. *J Orthop Res* (2011) **29**(12):1795–803. doi:10.1002/jor.21462
205. Lev DC, Kim SJ, Onn A, Stone V, Nam DH, Yazici S, et al. Inhibition of platelet-derived growth factor receptor signaling restricts the growth of human breast cancer in the bone of nude mice. *Clin Cancer Res* (2005) **11**(1):306–14.
206. Hwang RF, Yokoi K, Bucana CD, Tsan R, Killion JJ, Evans DB, et al. Inhibition of platelet-derived growth factor receptor phosphorylation by STI571 (Gleevec) reduces growth and metastasis of human pancreatic carcinoma in an orthotopic nude mouse model. *Clin Cancer Res* (2003) **9**(17):6534–44.
207. Mathew P, Thall PF, Bucana CD, Oh WK, Morris MJ, Jones DM, et al. Platelet-derived growth factor receptor inhibition and chemotherapy for castration-resistant prostate cancer with bone metastases. *Clin Cancer Res* (2007) **13**(19):5816–24. doi:10.1158/1078-0432.CCR-07-1269
208. Iqbal N, Iqbal N. Imatinib: a breakthrough of targeted therapy in cancer. *Chemother Res Pract* (2014) **2014**:357027. doi:10.1155/2014/357027
209. Erlebacher A, Derynck R. Increased expression of TGF-β2 in osteoblasts results in an osteoporosis-like phenotype. *J Cell Biol* (1996) **132**(1–2):195–210. doi:10.1083/jcb.132.1.195
210. Filvaroff E, Erlebacher A, Ye J, Gitelman SE, Lotz J, Heillman M, et al. Inhibition of TGF-beta receptor signaling in osteoblasts leads to decreased bone remodeling and increased trabecular bone mass. *Development* (1999) **126**(19):4267–79.
211. Johnson RW, Nguyen MP, Padalecki SS, Grubbs BG, Merkel AR, Oyajobi BO, et al. TGF-beta promotion of Gli2-induced expression of parathyroid hormone-related protein, an important osteolytic factor in bone metastasis, is independent of canonical Hedgehog signaling. *Cancer Res* (2011) **71**(3):822–31. doi:10.1158/0008-5472.CAN-10-2993
212. Buijs JT, Stayrook KR, Guise TA. TGF-beta in the Bone Microenvironment: Role in Breast Cancer Metastases. *Cancer Microenviron* (2011) **4**(3):261–81. doi:10.1007/s12307-011-0075-6
213. Kingsley LA, Fournier PG, Chirgwin JM, Guise TA. Molecular biology of bone metastasis. *Mol Cancer Ther* (2007) **6**(10):2609–17. doi:10.1158/1535-7163.MCT-07-0234
214. Ehata S, Hanyu A, Fujime M, Katsuno Y, Fukunaga E, Goto K, et al. Ki26894, a novel transforming growth factor-beta type I receptor kinase inhibitor, inhibits *in vitro* invasion and *in vivo* bone metastasis of a human breast cancer cell line. *Cancer Sci* (2007) **98**(1):127–33. doi:10.1111/j.1349-7006.2006.00357.x
215. Mohammad KS, Javelaud D, Fournier PG, Niewolna M, McKenna CR, Peng XH, et al. TGF-β-RI kinase inhibitor SD-208 reduces the development and progression of melanoma bone metastases. *Cancer Res* (2011) **71**(1):175–84. doi:10.1158/0008-5472.CAN-10-2651
216. Yin JJS, Selander K, Chirgwin JM, Dallas M, Grubbs BG, Wieser RM, et al. TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells

and bone metastases development. *J Clin Invest* (1999) **103**(2):197–206. doi:10.1172/JCI3523
217. Li X, Sterling JA, Fan KH, Vessella RL, Shyr Y, Hayward SW, et al. Loss of TGF-β responsiveness in prostate stromal cells alters chemokine levels and facilitates the development of mixed osteoblastic/osteolytic bone lesions. *Mol Cancer Res* (2012) **10**(4):494–503. doi:10.1158/1541-7786.MCR-11-0506
218. Furstenberger G, Senn HJ. Insulin-like growth factors and cancer. *Lancet Oncol* (2002) **3**(5):298–302. doi:10.1016/S1470-2045(02)00731-3
219. Grzmil M, Hemmerlein B, Thelen P, Schweyer S, Burfeind P. Blockade of the type I IGF receptor expression in human prostate cancer cells inhibits proliferation and invasion, up-regulates IGF binding protein-3, and suppresses MMP-2 expression. *J Pathol* (2004) **202**(1):50–9. doi:10.1002/path.1492
220. Zhao H, Dupont J, Yakar S, Karas M, LeRoith D. PTEN inhibits cell proliferation and induces apoptosis by downregulating cell surface IGF-IR expression in prostate cancer cells. *Oncogene* (2004) **23**(3):786–94. doi:10.1038/sj.onc.1207162
221. Hiraga T, Myoui A, Hashimoto N, Sasaki A, Hata K, Morita Y, et al. Bone-derived IGF mediates crosstalk between bone and breast cancer cells in bony metastases. *Cancer Res* (2012) **72**(16):4238–49. doi:10.1158/0008-5472.CAN-11-3061
222. Ryan CJ, Haqq CM, Simko J, Nonaka DF, Chan JM, Weinberg V, et al. Expression of insulin-like growth factor-1 receptor in local and metastatic prostate cancer. *Urol Oncol* (2007) **25**(2):134–40.
223. Kwabi-Addo B, Ozen M, Ittmann M. The role of fibroblast growth factors and their receptors in prostate cancer. *Endocr Relat Cancer* (2004) **11**(4):709–24. doi:10.1677/erc.1.00535
224. Abate-Shen C, Shen MM. FGF signaling in prostate tumorigenesis – new insights into epithelial-stromal interactions. *Cancer Cell* (2007) **12**(6):495–7. doi:10.1016/j.ccr.2007.11.021
225. Corn PG, Wang F, McKeehan WL, Navone N. Targeting fibroblast growth factor pathways in prostate cancer. *Clin Cancer Res* (2013) **19**(21):5856–66. doi:10.1158/1078-0432.CCR-13-1550
226. Dorkin TJ, Robinson MC, Marsh C, Neal DE, Leung HY. aFGF immunoreactivity in prostate cancer and its co-localization with bFGF and FGF8. *J Pathol* (1999) **189**(4):564–9. doi:10.1002/(SICI)1096-9896(199912)189:4<564::AID-PATH480>3.0.CO;2-1
227. Giri D, Ropiquet F, Ittmann M. Alterations in expression of basic fibroblast growth factor (FGF) 2 and its receptor FGFR-1 in human prostate cancer. *Clin Cancer Res* (1999) **5**(5):1063–71.
228. Ropiquet F, Giri D, Kwabi-Addo B, Mansukhani A, Ittmann M. Increased expression of fibroblast growth factor 6 in human prostatic intraepithelial neoplasia and prostate cancer. *Cancer Res* (2000) **60**(15):4245–50.
229. Freeman KW, Welm BE, Gangula RD, Rosen JM, Ittmann M, Greenberg NM, et al. Inducible prostate intraepithelial neoplasia with reversible hyperplasia in conditional FGFR1-expressing mice. *Cancer Res* (2003) **63**(23):8256–63.
230. Acevedo VD, Gangula RD, Freeman KW, Li R, Zhang Y, Wang F, et al. Inducible FGFR-1 activation leads to irreversible prostate adenocarcinoma and an epithelial-to-mesenchymal transition. *Cancer Cell* (2007) **12**(6):559–71. doi:10.1016/j.ccr.2007.11.004
231. Ozen M, Giri D, Ropiquet F, Mansukhani A, Ittmann M. Role of fibroblast growth factor receptor signaling in prostate cancer cell survival. *J Natl Cancer Inst* (2001) **93**(23):1783–90. doi:10.1093/jnci/93.23.1783
232. Li ZG, Mathew P, Yang J, Starbuck MW, Zurita AJ, Liu J, et al. Androgen receptor-negative human prostate cancer cells induce osteogenesis in mice through FGF9-mediated mechanisms. *J Clin Invest* (2008) **118**(8):2697–710. doi:10.1172/JCI33093
233. Valta MP, Hentunen T, Qu Q, Valve EM, Harjula A, Seppanen JA, et al. Regulation of osteoblast differentiation: a novel function for fibroblast growth factor 8. *Endocrinology* (2006) **147**(5):2171–82. doi:10.1210/en.2005-1502
234. Valta MP, Tuomela J, Bjartell A, Valve E, Vaananen HK, Harkonen P. FGF-8 is involved in bone metastasis of prostate cancer. *Int J Cancer* (2008) **123**(1):22–31. doi:10.1002/ijc.23422
235. Teishima J, Shoji K, Hayashi T, Miyamoto K, Ohara S, Matsubara A. Relationship between the localization of fibroblast growth factor 9 in prostate cancer cells and postoperative recurrence. *Prostate Cancer Prostatic Dis* (2012) **15**(1):8–14. doi:10.1038/pcan.2011.48
236. Lamb LE, Knudsen BS, Miranti CK. E-cadherin-mediated survival of androgen-receptor-expressing secretory prostate epithelial cells derived from a stratified *in vitro* differentiation model. *J Cell Sci* (2010) **123**(Pt 2):266–76. doi:10.1242/jcs.054502
237. Foster BA, Evangelou A, Gingrich JR, Kaplan PJ, DeMayo F, Greenberg NM. Enforced expression of FGF-7 promotes epithelial hyperplasia whereas a dominant negative FGFR2iiib promotes the emergence of neuroendocrine phenotype in prostate glands of transgenic mice. *Differentiation* (2002) **70**(9–10):624–32. doi:10.1046/j.1432-0436.2002.700915.x
238. Memarzadeh S, Xin L, Mulholland DJ, Mansukhani A, Wu H, Teitell MA, et al. Enhanced paracrine FGF10 expression promotes formation of multifocal prostate adenocarcinoma and an increase in epithelial androgen receptor. *Cancer Cell* (2007) **12**(6):572–85. doi:10.1016/j.ccr.2007.11.002
239. Liu W, Zhou Y, Reske SN, Shen C. PTEN mutation: many birds with one stone in tumorigenesis. *Anticancer Res* (2008) **28**(6A):3613–9.
240. Xu J, Wang R, Xie ZH, Odero-Marah V, Pathak S, Multani A, et al. Prostate cancer metastasis: role of the host microenvironment in promoting epithelial to mesenchymal transition and increased bone and adrenal gland metastasis. *Prostate* (2006) **66**(15):1664–73. doi:10.1002/pros.20488
241. Corey E, Quinn JE, Buhler KR, Nelson PS, Macoska JA, True LD, et al. LuCaP 35: a new model of prostate cancer progression to androgen independence. *Prostate* (2003) **55**(4):239–46. doi:10.1002/pros.10198
242. Corey E, Quinn JE, Vessella RL. A novel method of generating prostate cancer metastases from orthotopic implants. *Prostate* (2003) **56**(2):110–4. doi:10.1002/pros.10235

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Review Article

Molecular Mechanisms of Therapies for Prostate Cancer with Bone Metastasis

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Abstract

Prostate cancer (PCa) is one of the most diagnosed cancers in American men and is a leading cause of cancer deaths in advanced forms of disease. Seventy to ninety percent of men who die of PCa will have developed bone metastases during the course of the disease, and these bone metastases cause severe pain that can lead to further skeletal complications. The standard treatment for PCa generally includes androgen deprivation therapy, radiation, and chemotherapy, either singly or in combination, depending on disease progression. These treatments are able to slow disease progression slightly, but an urgent need remains for a more curative approach. In addition to the adverse effects resulting from these treatments, androgen deprivation therapy can often lead to a castration-resistant form of the disease, which is even more difficult to treat and often becomes metastatic. In the past several years, new drugs that target androgens, the bone microenvironment, and other mechanisms involved in PCa have shown promise in clinical trials for advanced PCa; these include abiraterone, enzalutamide, sipuleucel-T, radium-223, denosumab, and cabazitaxel, and will be described in further detail in this review. The continuous improvement upon current therapies and development of new drugs is promising for the future of advanced PCa, and the authors will give their perspective on these different treatment approaches.

Introduction

According to the American Cancer Society, 1 in 7 men will be diagnosed with prostate cancer (PCa) in their lifetime.[1] There is a 10-year survival rate of 99% when the disease is caught early; however, this rate drops to 30% when patients develop metastasis. Seventy to ninety percent of patients who die of PCa have bone metastasis, which classifies their disease state as advanced.[2] PCa bone metastasis causes skeletal-related events (SREs), such as fracture, hypercalcemia, or spinal cord compression, as well as severe pain. Current therapies, such as bisphosphonates, steroids, and chemotherapies, are used to relieve these symptoms and slow disease progression, but they are also associated with adverse reactions and do not significantly improve the patients' overall survival. Therefore, developing new approaches to target PCa bone metastasis is an urgent need.

It is important to understand the molecular mechanisms behind PCa metastasis when developing new therapies. Normal bone homeostasis is largely dependent on osteoclast and osteoblast function during bone turnover, as well as the various factors that are released during the process. PCa cells secrete factors such as parathyroid hormone-related protein (PTHrP), which initiates osteoblast expression of the receptor activator of nuclear factor kappa-B ligand (RANKL), which further stimulates maturation of osteoclasts to resorb bone. The bone is a powerhouse, which stores many growth factors and cytokines that are released upon PCa-induced resorption. Factors like transforming growth factor-beta (TGF-β) are released from the bone matrix and cause stimulation of osteoblasts, which, in turn, signal to osteoclasts for more bone resorption. This rapid bone turnover that occurs during PCa bone metastasis is known as the "vicious cycle".

This review will discuss the molecular mechanisms of current clinical therapies and our perspectives on the potential new therapies for PCa bone metastasis patients. There have been several drugs approved for PCa therapy in the last few years, targeting a variety of cellular pathways. We will focus on the main approaches to current PCa therapy, primarily on the respective underlying molecular mechanisms of each treatment. A list of drugs discussed in this review along with their respective categories and mechanisms of action are included in Table 1.

Targeting cancer cells

Androgen ablation therapy

The surgical or chemical removal of androgen signaling, known as androgen deprivation therapy (ADT), is a common treatment for advanced PCa. ADT reduces the amount of available androgen that would otherwise stimulate PCa cell growth through the androgen receptor (AR). Administration of ADT prior to primary tumor removal is still controversial, but studies have shown that it leads to a decrease in lymph node invasion and better organ confinement as well as overall progression-free survival when initiated at diagnosis.[3] Some clinicians choose a combination treatment of pelvic

Keywords: Prostate cancer; Bone metastasis; Treatment; Androgen deprivation therapy; Radiation; Bone microenvironment; Androgen; Castration-resistant.

Abbreviations: PCa, prostate cancer; ADT, androgen deprivation therapy; AR, androgen receptor; CRPC, castration-resistant prostate cancer; LHRH, luteinizing hormone-releasing hormone; PSA, prostate-specific antigen; RANKL, receptor activator of nuclear factor kappa-B ligand; U.S. FDA, United States Food and Drug Administration; SREs, skeletal-related events.

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Table 1. Mechanistic actions of prostate cancer therapeutics

Drug Name	Therapeutic Category	Mechanism of Action
Abiraterone	Androgen Deprivation Therapy	CYP17 inhibitor that decreases circulating levels of androgen
Cabitaxel	Chemotherapeutic	Antimitotic that stops PCa division and alters the cell efflux pump to extend cellular drug levels
CAR T Therapy	Immune Therapy	Targets prostate-specific antigens such as PSMA or PSCA to kill PCa cells
Degarelix	LHRH Antagonist	Stops LHRH from binding its receptor and prevents LH flare that would otherwise spike testosterone levels
Denosumab	RANKL Inhibitor	RANKL antibody that blocks osteoclast differentiation and activity
Docetaxel	Chemotherapeutic	Antimitotic agent that stops PCa cell division
Enzalutamide	Androgen Deprivation Therapy	Androgen receptor inhibitor that prevents AR translocation to the nucleus and further androgen signaling
Galeterone and Niclosamide	Androgen Deprivation Therapy	Targets AR-V7 to reduce androgen levels (generally used in combination with either abiraterone or enzalutamide)
Ipilimumab	Immune Checkpoint Therapy	CTLA-4 antibody that promotes adaptive immunity and T cell response to attack PCa cells
Nivolumab and Pembrolizumab	Immune Checkpoint Therapy	PD-1 antibody that allows immune response of cytotoxic T cells to attack PCa cells
Radium-223	Radiation Therapy	Alpha-emitting radiation with structure similar to calcium for greater bone specificity
Sipuleucel-T	Immunotherapy	Cancer vaccine that facilitates the T cell immune response targeting PAP on PCa cells
SRPK1 Inhibitor	VEGF and FGFR Inhibitors	Alters VEGF splicing to antiangiogenic isoforms, reducing tumor angiogenesis
Zoledronic Acid	Bisphosphonate	Targets osteoclasts to reduce bone resorption and turnover

radiation and ADT for their patients to target any micrometastases that may be present at the time of primary tumor removal.[4]

One disadvantage to ADT is that a flare reaction in bone metastasis can occur upon initiation, leading to rapid bone repair and an increase in osteoblast activity.[5] This makes it very important for clinicians to take into account the progression of disease and risk for the patient before beginning treatment. While ADT can slow progression of disease, there are adverse effects for patients, including hot flashes, night sweats, hair loss, irritability, loss of libido, and erectile dysfunction.[6] Another disadvantage to ADT is that testosterone levels have to be monitored consistently, which becomes difficult when trying to detect castration levels of 50 ng/dL or less. The testosterone level of 50 ng/dL has been set as the standard for castration, but there is question as to whether the baseline should be reduced to 20 ng/dL, for which better patient results have been reported.[3]

Drugs like abiraterone, enzalutamide, and galeterone, have been developed to target alternate androgen activation of AR that may contribute to rising testosterone levels and PCa progression.[7–9] The first of these drugs, abiraterone, is a CYP17 inhibitor that prevents the conversion of pregnenalone to dihydrotestosterone (DHT) and has been shown to significantly increase survival, particularly in patients with metastatic castration-resistant PCa (CRPC), as compared to treatment with prednisone alone.[9] The second of these drugs, enzalutamide, works through an AR binding competition mechanism and inhibition of translocation of AR into the nucleus, both of which impair AR activation.[8] Both abiraterone and enzalutamide have improved survival in patients, but one-third of PCa patients have an AR-V7 splice variant that makes them resistant to this therapy. In order to overcome resistance to these drugs, a couple of new therapies are in trials, including niclosamide and galeterone, and have shown some success by specifically targeting AR-V7 along with inhibition of AR by abiraterone or enzalutamide.[10] These alternatives could be used to reduce androgen signaling ever further, but, even so, ADT does not cure prostate cancer and can lead to castration-resistant disease.

Luteinizing hormone-releasing hormone (LHRH) agonists and antagonists are another way to reduce androgen signaling in PCa patients. LHRH agonists act on the anterior pituitary gland to block testosterone production.[3] The down side of this agonist is that it causes an initial spike in LH release that can stimulate PCa cells, leading to side effects like spinal cord compression, ureteral/urethral obstruction, or bone pain. In this case, antiandrogens are generally added to the therapy to block downstream AR signaling and suppress these side effects. This strategy of combined androgen blockade is used to keep the levels of androgens below the castration baseline of 50 ng/dL, even with the LH burst from drug initiation.

Combined therapies can help keep testosterone levels at bay but come with added risk to patients, including increased risk for diabetes, osteoporosis, cardiac events and stroke.[3] LHRH antagonists have been more recently investigated for their ability to help avoid LH flare from the pituitary gland, meaning that no antiandrogens would be needed to counteract the increase in testosterone and ultimately equating to less risk for adverse effects. Degarelix is an LHRH antagonist that works by consistently suppressing follicle-stimulating hormone (FSH) levels, which would otherwise stimulate PCa growth. LHRH antagonists have been found to improve progression-free survival better than other ADTs as well.[3]

While these therapies can reduce androgen signaling to PCa cells and slow down progression of disease, patients and clinicians have to decide on the best treatment for their individual case. Since ADT can lead to CRPC and metastasis, a patient with low-risk disease has to acknowledge the potential for future complications

associated with starting ADT. Once PCa becomes castration-resistant or metastatic, it is very difficult to treat and most often leads to patient death.

Chemotherapy

Taxanes are a group of chemotherapeutic agents used to treat advanced PCa that interfere with microtubules during mitosis and prevent the cell from reaching anaphase, thereby leading to apoptosis.[11] Docetaxel was approved for treatment of metastatic CRPC in 2004 and has since become the standard treatment for advanced disease, but, unfortunately, disease progression is still inevitable. Docetaxel used in combination with other therapies has provided some promising results of progression-free survival.[7,11,12] Even so, some PCa becomes resistant to docetaxel and new treatment options are needed. Cabazitaxel was approved by the U.S. Food and Drug Administration (FDA) to treat docetaxel-resistant PCa, and works by targeting microtubules, altering the efflux pump in the cell (so that drug remains in the cell longer), and inducing apoptosis.[12] Carboplatin (a platinum-based drug), and everolimus (an mTOR inhibitor) have been used in clinical trials as a combination therapy and have been shown to exert antitumor effects and to reduce prostate-specific antigen (PSA) levels.[7]

Chemotherapy agents such as docetaxel can be beneficial for many patients with metastatic CRPC; however, more specific bone-targeting agents could help eliminate the adverse effects to other non-cancerous replicating cells throughout the patient's body. In general, docetaxel is well tolerated by patients, but it can result in fever, fatigue, pneumonitis, gastrointestinal complications, neuropathies and more.[13]

Immune therapy

Sipuleucel-T

Sipuleucel-T has recently been approved by the U.S. FDA as an antigen presenting cell (APC)-based vaccine for PCa.[6] Sipuleucel-T works through T cell immunity targeted against PCa cells, and utilizes peripheral blood mononuclear cells, including APCs, from the patient. These cells are activated *ex vivo* with a recombinant fusion protein (PA2024) consisting of a prostate antigen, prostatic acid phosphatase (PAP), fused to the granulocyte-macrophage colony-stimulating factor. This therapy acts through APC stimulation of T cell immune responses targeted to PAP that is highly expressed on most PCa cells. The prostate antigen is incubated with patient APCs (obtained by blood draw) and infused back into the patient once the cells are activated and ready to fight the PCa cells.

Sipuleucel-T has been shown to reduce the risk of death among metastatic CRPC patients and to increase the median survival by at least 4 months, as compared to other currently available therapeutics.[14,15] Adverse effects for this immunotherapy are similar to flu-like symptoms, and include headache, fever, nausea, chills, joint pain, and some gastrointestinal discomfort. But unlike with the other treatments, these symptoms usually occur immediately following application of the drug and rescind within a couple of days. The extended survival (of at least 4 months as compared to the other drugs), the better quality of life during treatment, and the reduced risk of death by 22.5% could make this therapy worth the time and money for many patients with advanced or metastatic CRPC.[15]

Immune checkpoint therapy

Immune checkpoint therapy is another treatment method that has been in clinical trials for multiple cancers, including PCa. These kinds of therapies utilize various antibody targets involved in immune response to elicit antitumor effects through T cells.[16] Nivolumab and pembrolizumab are antibodies against the programmed death-1 (PD-1) protein that promote cytotoxic T cell immunity to increase the attack on and destruction of cancer cells. Ipilimumab is another immune checkpoint therapy that has been in clinical trials, which targets the cytotoxic T lymphocyte-associated protein 4 (CTLA-4). This therapy works similarly by inducing adaptive immunity so that T cells become activated and begin attacking PCa cells.[16,17]

Chimeric antigen receptor (CAR) T cell therapy

CAR T cell therapy is another novel treatment used in various cancer types. This type of therapy targets cancer-specific antigens in order to gain specificity and kill cancer cells solely. The prostate-specific membrane antigen (PSMA) and prostate stem cell antigen (PSCA) have been targeted in clinical trials for PCa, but have shown minimal antitumor effects.[18]

More advanced CAR T cell therapies use a combination of costimulatory agents, and these have shown much greater antitumor effects than the first-generation therapies; however, they have also shown a greater potential for eliciting more severe adverse immune reactions. Cytokine release syndrome remains a concern for CAR T cell therapy, as it can lead to fevers, fatigue, nausea, and the life-threatening conditions of multi-organ dysfunction or failure.[19,20] PCa expresses many antigens that are not present in other tissues, which is what makes this type of therapy so attractive, and once the adverse effects of these drugs are diminished, the development of combined immunotherapies targeting these antigens will have substantial antitumor potential for both metastatic and castration-resistant disease.

Targeting the bone microenvironment

Bisphosphonates

While chemotherapies, ADT, and radiation may help reduce progression of disease, there is still need for therapy that can treat bone metastasis specifically. There are currently several drugs available that target the bone microenvironment so that normal bone mass can be achieved.

Bisphosphonates are synthetic analogues of phosphates that inhibit hydroxyapatite crystal dissolution in bone tissues. These drugs are endocytosed by osteoclasts and become incorporated into the cell as non-hydrolyzable analogues of adenosine triphosphate (ATP), which accumulates in the cell, inhibits absorption, and induces apoptosis. Targeting osteoclasts leads to reduction in bone resorption and decreased bone turnover, which is increased in metastases. There are 3 different classes of bisphosphonates used in the clinic, and the second- and third-generations include nitrogen-containing bisphosphonates, like zoledronic acid.

Zoledronic acid was found to be 10,000-fold more potent than first-generation bisphosphonates, such as clodronate.[21] Zoledronic acid improves upon other bisphosphonates by additionally inhibiting farnesyl diphosphate synthase, which blocks the mevalatone pathway. This reduces downstream metabolites and prevents post-

translational modifications of GTPases like Ras, Rab, Rho, and Rac that are essential signaling proteins for regulation of multiple cell processes in osteoclasts.[21] Bisphosphonates bind to the bone matrix and are absorbed by osteoclasts during bone remodeling that occurs during metastasis, and the interference with their maturation leads to apoptosis. This reduces osteoclast function and inhibits resorption, leading to overall reduction in bone turnover, osteoblast function, and bone mass maintenance.[22,23] While the bisphosphonates can help slow disease progression and reduce bone pain, there still remains a need for more curative treatment.

RANKL inhibitors

Denosumab has been developed as an alternative therapy to bisphosphonates, and functions by inhibiting RANK signaling. It is a monoclonal antibody to RANK ligand (RANKL), and has been shown to increase time to skeletal-related events (SREs) better than bisphosphonates.[24] Blocking RANKL prevents mature osteoclast activity as well as their differentiation, which interrupts bone turnover.[21] Denosumab was found to be a superior treatment to zoledronic acid for time to first SRE, with the time being increased 18% (36 months), but without any significant change in overall survival or disease progression. It did, however, decrease serum levels of urine N-telopeptide (uNTx) and alkaline phosphatase (ALP) as compared to zoledronic acid treatment. uNTx is a bone turnover marker that has been associated with increased risk in patients with PCa. In particular, patients with moderate to high levels of uNTx were shown to have a 2-fold increased risk for skeletal complications and disease progression; thus, a drug that results in lower levels of this marker is expected to significantly reduce risk for PCa advancement.[21,25]

There are particular benefits to the two bone-targeted therapies described above, and each patient will have a different case that may benefit from either one. Zoledronic acid needs to be intravenously administered and patients are monitored for renal complications throughout treatment. Denosumab can be given subcutaneously or orally and has no adverse effects in renal health or acute phase reactions. Patients will need to consider the cost of treatment along with long-term adverse effects and benefits depending on their disease severity and age.

Targeting PCa and bone microenvironment

Radiation

Radiation therapy after radical prostatectomy is standard of care for high-risk and metastatic PCa.[3] Radiation localized to the pelvic region, as mentioned previously, is an option for patients that may be at risk for micrometastases at time of diagnosis. This can help prevent further growth in the pelvis as well as potential spread to other bones. There has been evidence to suggest that radiation not only treats PCa metastases, but also affects the bone microenvironment so that the bone is no longer hospitable to metastasis. Treatment with radiopharmaceuticals could potentially keep PCa cells or bone metastases dormant by inhibiting the necessary bone factors that lead to disease progression.[5] This treatment can be a useful tool for CRPC by targeting mechanisms other than AR signaling, but other damaging effects will occur with treatment.

Radium-223 is an alpha emitting radiopharmaceutical used for PCa bone metastasis. It has a structure similar to calcium that helps it target the bone microenvironment and is less damaging than beta or gamma emissions.[24,26] A significant improvement in overall survival of at least 4 months has been seen in CRPC bone metastasis patients.[24] Multiple studies have been performed with this therapy, and have produced mixed results as to whether survival and disease progression are affected, but, overall, patients that have aggressive CRPC with a greater number of bone metastases have longer progression-free survival than those without Radium-223 treatment.[5] Based on these results, it seems that this therapy may be a good choice for patients with aggressive PCa that has progressed to CRPC with multiple metastases.

Conclusion and perspectives

The continued development of new treatments for advanced PCa is promising for the future of patient therapy. Combination therapies targeted to the bone as well as new immunotherapies, such as sipuleucel-T and CAR T cell therapy have the potential to significantly reduce disease progression in PCa patients. Two of the biggest hurdles yet to be overcome are the adverse reactions that result from these drugs and the need to find a way by which to determine which therapy is best for each specific case.

There are currently 90 phase I/II trials and several phase III trials available for PCa patients.[27] Vascular endothelial growth factor (VEGF) and fibroblast growth factor receptor (FGFR) inhibitors are a recent development designed to target angiogenesis in PCa tumors. A serine-arginine protein kinase 1 (SRPK1) inhibitor that switches VEGF splicing to antiangiogenic isoforms, rather than angiogenic ones, has shown improvement in PCa patient-derived xenograft models. This inhibitor has reduced tumor growth and microvessel formation in mouse tumors, and the SR proteins that the inhibitor regulates are up-regulated in human PCa tissues in correlation with disease stage and invasion. Drugs like alibercept, pasopanib, dovitinib, and lucitanib are currently in clinical trials, targeting VEGF and FGF pathways, but have not shown much improvement over the current therapies as of yet.[28]

There are many pathways and possible targets for PCa bone metastasis, and after reviewing the past and current therapies, combination treatment seems to be the most aggressive and successful treatment method for advanced and metastatic CRPC. Although combination therapy can have adverse side effects, the patients must compare the quality of life they expect during therapy to the expected survival and potential for disease progression. Each patient will present a different case, and depending on age and disease severity, clinicians will have to decide which therapy would be more beneficial for each one. With the new therapies that have been approved in the past several years and the treatments continuously entering the pipeline, there is hope for the future of metastatic CRPC therapy. But, as the current drugs only prolong the inevitable, there is still a need for a more curative treatment approach.

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Conception and design (AVA, XL), writing of the manuscript (AVA, EW, XL), supervision (XL).

References

[1] Li X, Sterling JA, Fan KH, Vessella RL, Shyr Y, Hayward SW, *et al*. Loss of TGF-beta responsiveness in prostate stromal cells alters chemokine levels and facilitates the development of mixed osteoblastic/osteolytic bone lesions. Mol Cancer Res 2012;10(4):494–503. doi:10.1158/1541-7786.

[2] Canalichio K, Jaber Y, Wang R. Surgery and hormonal treatment for prostate cancer and sexual function. Transl Androl Urol 2015;4(2):103–109. doi:10.3978/j.issn.2223-4683.2015.01.13.

[3] Rove KO, Crawford ED. Traditional androgen ablation approaches to advanced prostate cancer: new insights. Can J Urol 2014;21(2 Supp 1):14–21.

[4] Juloori A, Shah C, Stephans K, Vassil A, Tendulkar R. Evolving Paradigm of Radiotherapy for High-Risk Prostate Cancer: Current Consensus and Continuing Controversies. Prostate Cancer 2016;2016:2420786. doi:10.1155/2016/2420786.

[5] Bilen MA, Johnson MM, Mathew P, Pagliaro LC, Araujo JC, Aparicio A, *et al*. Randomized phase 2 study of bone-targeted therapy containing strontium-89 in advanced castrate-sensitive prostate cancer. Cancer 2015;121(1):69–76. doi:10.1002/cncr.28971.

[6] DeSantis CE, Lin CC, Mariotto AB, Siegel RL, Stein KD, Kramer JL, *et al*. Cancer treatment and survivorship statistics, 2014. CA Cancer J Clin 2014;64(4):252–271. doi:10.3322/caac.21235.

[7] Yoo S, Choi SY, You D, Kim CS. New drugs in prostate cancer. Prostate Int 2016;4(2):37–42. doi:10.1016/j.prnil.2016.05.001.

[8] Antonarakis ES, Lu C, Wang H, Luber B, Nakazawa M, Roeser JC, *et al*. AR-V7 and resistance to enzalutamide and abiraterone in prostate cancer. N Engl J Med 2014;371(11):1028–1038. doi:10.1056/NEJMoa1315815.

[9] Ryan CJ, Smith MR, Fizazi K, Saad F, Mulders PF, Sternberg CN, *et al*. Abiraterone acetate plus prednisone versus placebo plus prednisone in chemotherapy-naive men with metastatic castration-resistant prostate cancer (COU-AA-302): final overall survival analysis of a randomised, double-blind, placebo-controlled phase 3 study. Lancet Oncol 2015;16(2):152–160. doi:10.1016/S1470-2045(14)71205-7.

[10] Liu C, Armstrong C, Zhu Y, Lou W, Gao AC. Niclosamide enhances abiraterone treatment via inhibition of androgen receptor variants in castration resistant prostate cancer. Oncotarget 2016;7(22):32210–32220. doi:10.18632/oncotarget.8493.

[11] Chandrasekar T, Yang JC, Gao AC, Evans CP. Mechanisms of resistance in castration-resistant prostate cancer (CRPC). Transl Androl Urol 2015;4(3):365–380. doi:10.3978/j.issn.2223-4683.2015.05.02.

[12] Tsao CK, Cutting E, Martin J, Oh WK. The role of cabazitaxel in the treatment of metastatic castration-resistant prostate cancer. Ther Adv Urol 2014;6(3):97–104. doi:10.1177/1756287214528557.

[13] Ho MY, Mackey JR. Presentation and management of docetaxel-related adverse effects in patients with breast cancer. Cancer Manag Res 2014;6:253–259. doi:10.2147/CMAR.S40601.

[14] Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF, *et al*. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. N Engl J Med 2010;363(5):411–422. doi:10.1056/NEJMoa1001294.

[15] Anassi E, Ndefo UA. Sipuleucel-T (provenge) injection: the first immunotherapy agent (vaccine) for hormone-refractory prostate cancer. P T 2011;36(4):197–202.

[16] Sharma P, Allison JP. Immune checkpoint targeting in cancer therapy: toward combination strategies with curative potential. Cell 2015;161(2):205–214. doi:10.1016/j.cell.2015.03.030.

[17] Carlino MS, Long GV. Ipilimumab Combined with Nivolumab: A Standard of Care for the Treatment of Advanced Melanoma?. Clin Cancer Res 2016;22(16):3992–3998. doi:10.1158/1078-0432.CCR-15-2944.

[18] Hillerdal V, Essand M. Chimeric antigen receptor-engineered T cells for the treatment of metastatic prostate cancer. BioDrugs 2015;29(2):75–89. doi:10.1007/s40259-015-0122-9.

[19] Kloss CC, Condomines M, Cartellieri M, Bachmann M, Sadelain M. Combinatorial antigen recognition with balanced signaling promotes selective tumor eradication by engineered T cells. Nat Biotechnol 2013;31(1):71–75. doi:10.1038/nbt.2459.

[20] Maude SL, Barrett D, Teachey DT, Grupp SA. Managing cytokine release syndrome associated with novel T cell-engaging therapies. Cancer J 2014;20(2):119–122. doi:10.1097/PPO.0000000000000035.

[21] Gampenrieder SP, Rinnerthaler G, Greil R. Bone-targeted therapy in metastatic breast cancer - all well-established knowledge?. Breast Care (Basel) 2014;9(5):323–330. doi:10.1159/000368710.

[22] El-Amm J, Aragon-Ching JB. Targeting Bone Metastases in Metastatic Castration-Resistant Prostate Cancer. Clin Med Insights Oncol 2016(Suppl 1):11–19. doi:10.4137/CMO.S30751.

[23] Ottanelli S. Prevention and treatment of bone fragility in cancer patient. Clin Cases Miner Bone Metab 2015;12(2):116–129. doi:10.11138/ccmbm/2015.12.2.116.

[24] Cook LM, Shay G, Araujo A, Lynch CC. Integrating new discoveries into the "vicious cycle" paradigm of prostate to bone metastases. Cancer Metastasis Rev 2014;33(2-3):511–525. doi:10.1007/s10555-014-9494-4.

[25] Fizazi K, Carducci M, Smith M, Damiao R, Brown J, Karsh L, *et al*. Denosumab versus zoledronic acid for treatment of bone metastases in men with castration-resistant prostate cancer: a randomised, double-blind study. Lancet 2011;377(9768):813–822. doi:10.1016/S0140-6736(10)62344-6.

[26] Sridhar SS, Joshua AM, Gregg R, Booth CM, Murray N, Golubovic J, *et al*. A phase II study of GW786034 (pazopanib) with or without bicalutamide in patients with castration-resistant prostate cancer. Clin Genitourin Cancer 2015;13(2):124–129. doi:10.1016/j.clgc.2014.06.001.

[27] Prostate Cancer Foundation. Available from: www.pcf.org. Accessed August 2016.

[28] Mavrou A, Oltean S. SRPK1 inhibition in prostate cancer: A novel anti-angiogenic treatment through modulation of VEGF alternative splicing. Pharmacol Res 2016;107:276–281. doi:10.1016/j.phrs.2016.03.013.

TGF-β signaling in osteoblasts inhibits prostate cancer bone metastasis

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Running Title: Loss of TβRII in osteoblasts promotes metastasis through bFGF

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Abstract

TβRII was undetectable by immunohistochemistry in either all or some of the cancer-associated osteoblast (CAOB) population in 92.5% of the prostate cancer (PCa) patient bone metastatic tissues examined. Using bone cell–specific *Tgfbr2* knock-out mouse models, we found that deletion of *Tgfbr2* in osteoblasts ($Tgfbr2^{Col1CreERT}$ KO) promoted, but in osteoclasts ($Tgfbr2^{LysMCre}$ KO) inhibited, PCa bone lesion development. bFGF was identified as the functional mediator in this lesion development by stimulating osteoclastogenesis, inhibiting osteoblastogenesis, and indirectly promoting tumor cell proliferation. Co-culture with PCa cells, *Tgfbr2* KO osteoblasts had increases of bFGF, parathyroid hormone receptor (PTH1R) and pCREB, and these increases were abolished by blocking PTHrP in the co-cultures. These data suggest that loss of TGF-β signalling in osteoblasts resulted an increase of PTH1R, which was stimulated by PC3-derived PTHrP and activated the downstream PKA pathway to up-regulate the expression of bFGF. bFGF, in turn promoted bone lesion development.

Significance: Our study highlights the role and mechanism of osteoblast-specific TβRII in PCa bone metastasis. Loss of TβRII results in increased PTH1R/pKA signaling, which up-regulates bFGF, making bFGF a promising therapeutic target in PCa bone metastasis by inhibiting both tumor growth and bone resorption.

Key words: prostate cancer, bone metastasis, transforming growth factor-β (TGF-β), TGF-β type II receptor (TβRII), bFGF, parathyroid hormone–related protein (PTHrP), PTH/PTHrP receptor (PTH1R), pCREB, PKA

Introduction

Prostate cancer (PCa) is the most common malignant disease and the second-leading cause of cancer-related death in men in the United States. Bone metastases have been found in 70-90% of the patients who die of PCa (1). Bone lesions develop through the interactions between disseminated PCa cells and the bone microenvironment. Two major bone cell types are involved in such interactions: osteoclasts, which are cells of myeloid lineage, and osteoblasts, which are cells of mesenchymal lineage. It is known that metastatic cancer cells secrete factors such as parathyroid hormone-related protein (PTHrP) that regulate osteoblasts to stimulate the maturation of osteoclasts. Osteoclasts resorb bone and release growth factors such as transforming growth factor beta (TGF-β) from the bone matrix. TGF-β further promotes tumor growth and bone destruction (2-12). PTHrP and TGF-β play central roles in the establishment and progression of bone metastases, but blocking either of them or their downstream signaling has not succeeded as a therapy.

TGF-β signaling starts with ligand binding to the TGF-β type II receptor (TβRII), which phosphorylates the TGF-β type I receptor, activating the downstream Smad pathway. We have found that TβRII in cancer-associated fibroblasts (CAFs) is lost in 70% of PCa patient primary samples (13) and in seven out of nine PCa bone metastatic tissues (14). The deletion of the TβRII gene (*TGFBR2*) in the prostate fibroblasts induces primary PCa tumor growth (13), castration resistance (15), and PCa cell adhesion to the bone matrix (14). However, the expression of TβRII in cells of the bone microenvironment and its role(s) in PCa bone lesion development are unknown. This has been studied in $Tgfbr2^{Col1CreERT}$ knock-out (KO) or $Tgfbr2^{LysMCre}$ KO mouse models.

Tgfbr2^{Col1CreERT} KO mice have loss of TGF-β signaling through the deletion of *Tgfbr2* (16) only in osteoblasts and fibroblasts by activation of *CreERT* driven by the collagen 1α2 (*Col1α2)* promoter (17). *Tgfbr2*^{LysMCre} KO mice have a deletion of *Tgfbr2* in myeloid lineage cells such as osteoclasts (18). We found that PCa bone lesion development induced by PC3 or DU145 cells was significantly promoted in *Tgfbr2*^{Col1CreERT} KO mice but was inhibited in *Tgfbr2*^{LysMCre} KO mice, relative to their respective *Tgfbr2*^{FloxE2} littermates.

In PCa patient bone metastatic tissues, TβRII expression was undetectable by immunohistochemistry in either all or some of the CAOB population of each sample. Therefore, we focused on the *Tgfbr2*^{Col1CreERT} KO mouse model, where the loss of TβRII is in osteoblasts. We found that basic fibroblast growth factor (bFGF) expression was up-regulated in CAOBs and that it mediated the increased PC3 bone lesion development in *Tgfbr2*^{Col1CreERT} KO mice relative to *Tgfbr2*^{FloxE2} mice. Functionally, bFGF mediates PC3-induced bone lesion development through indirect stimulation of tumor cell proliferation, direct promotion of osteoclastogenesis, angiogenesis, and CAF formation, and direct inhibition of osteoblastogenesis. Therefore, bFGF is a potential target for PCa bone metastasis. Furthermore, we found bFGF was up-regulated at least partly by the increased activation of PTH1R through downstream PKA signaling in CAOBs that had loss of TβRII.

Results

Loss of TβRII expression in cancer-associated osteoblasts of PCa patients bone metastasis tissues

In order to determine TβRII expression in bone cells, we used immunohistochemical analysis of a PCa patient bone metastasis tissue microarray. Focusing on the cancer-associated cells in the bone microenvironment, we identified 40 bone metastasis tissues in which cancer-associated osteoblasts (CAOBs) were present, based on their morphology and location. Specifically, these cells were defined as being grouped along the surface of the bone and were cuboidal or columnar with a central nucleus (18). Among these 40 tissues, TβRII expression was undetectable in either all or some of the CAOBs in each of thirty-seven tissues; TβRII in all of the CAOBs was found only in three tissues (**Figure 1A-D**). A positive association was found between TβRII expression in cancer-associated fibroblasts (CAFs) and CAOBs ($P = 0.002$), but not between CAOBs and tumor cells (**Figure 1E**). Further, expression of TβRII was detected in PCa cells from 122 out of 141 tissue samples; the average staining intensity was significantly higher in tumor cells from bone metastases than in those from visceral metastases ($P < 0.001$) (**Figure 1F**).

TβRII expression in CAOBs was positively associated with patients that had prostatectomy, treatment with Zometa, months of treatment with enzalutamide or abiraterone, and one or more years of survival from first bone metastasis. This association suggests that these clinical parameters may be predictors of increased TβRII expression. On the other hand, TβRII expression in CAOBs was negatively associated with months of treatment with ketoconazole or carbonplatin, treatment with other

bisphosphonates, and years of bone metastasis delay from initial diagnosis. This suggests that these clinical factors are predictors of decreased TβRII expression (**Supplemental Table 1**).

Together, these studies suggest that TβRII expression is frequently lost in the CAOBs in PCa patient bone metastatic sites and that certain clinical treatments are potential indicators for the expression of TβRII in CAOBs. On the other hand, PCa cells express higher TβRII levels in bone metastases relative to those growing in soft organs.

TGF-β signaling in osteoblasts inhibited, but in osteoclasts promoted, PCa bone metastasis

To investigate the role of bone cell–specific TGF-β signaling, PC3 or DU145 cells were intratibially injected into *Tgfbr2*^{Col1CreERT} KO mice, *Tgfbr2*^{LysMCre} KO mice, or their respective *Tgfbr2*^{FloxE2} littermates. *Tgfbr2*^{LysMCre} KO mice have been characterized and a normal bone phenotype was identified, as we previously published (18). We therefore first compared the bones between *Tgfbr2*^{Co1lCreERT} KO and *Tgfbr2*^{FloxE2} littermates. Cell-specific *Cre* expression and activation were confirmed by genotyping and immunohistochemical (IHC) analysis of GFP expression in *Tgfbr2*^{Col1CreERT} KO mouse tibiae (**Supplemental Figure 1A**). p-Smad2, the TGF-β signaling downstream transcriptional factor, was also confirmed by IHC to be expressed in osteoblasts from *Tgfbr2*^{FloxE2} mice but not from *Tgfbr2*^{Col1CreERT} KO mice. Loss of p-Smad2 was confirmed in the GFP-positive *Tgfbr2*^{Col1CreERT} KO osteoblasts by IHC in the serial sections, but was not seen in the *Tgfbr2*^{FloxE2} osteoblasts (**Supplemental Figure 1A**). After confirming the loss of TGF-β signaling in the osteoblasts of *Tgfbr2*^{Col1CreERT} KO mice, their phenotypes

were evaluated and found to be normal relative to *Tgfbr2*^{FloxE2} littermates. We analyzed the bones by histomorphometry (**Supplemental Figure 1B**), whole body X-rays, and microcomputed tomography (μCT) (**Supplemental Figure 2**).

The PCa-induced bone lesions were imaged by weekly X-rays, and bone lesion areas were measured and quantified using Metamorph software. The average total area of PC3 osteolytic lesions in *Tgfbr2*^{Col1CreERT} KO mice increased by roughly 0.01 cm^3 more per week than those in *Tgfbr2*^{FloxE2} mice. Significant differences in bone lesion areas were detected in the second, third and fourth week after injection (**Figure 2A-C**). Bone lesion incidence rates were not different between the *Tgfbr2*^{Col1CreERT} KO and *Tgfbr2*^{FloxE2} mice.

Contrary to these results, PC3-induced bone lesions were significantly smaller in *Tgfbr2*^{LysMCre} KO mice relative to their respective *Tgfbr2*^{FloxE2} littermates at the third and fourth week after injection ($P < 0.001$, **Figure 2D-F**). PC3 osteolytic lesions in *Tgfbr2*^{LysMCre} KO mice were inhibited by roughly 0.01 cm^3 per week relative to *Tgfbr2*^{FloxE2} mice.

Similar effects on lesion sizes were observed in DU145-induced osteolytic/osteoblastic bone lesions; total lesion area increased by approximately 0.01 cm^3 more per week in DU145-injected tibiae from *Tgfbr2*^{Col1CreERT} KO mice relative to those from *Tgfbr2*^{FloxE2} mice. Differences in total bone lesion area were significant the third week after injections ($P < 0.001$, **Supplemental Figure 3A and 3C**). Tumor incidence rates differed significantly between DU145-injected *Tgfbr2*^{Col1CreERT} KO mice and *Tgfbr2*^{FloxE2} mice as well: the median time until a tumor lesion could be detected was

3 weeks for *Tgfbr2*^{Col1CreERT} KO mice and 5 weeks for *Tgfbr2*^{FloxE2} mice ($P < 0.01$, **Supplemental Figure 3B**).

Together, these data suggest that TGF-β signaling in osteoblasts inhibited, but in osteoclasts promoted, PCa bone lesion development.

Tgfbr2^{Col1CreERT} KO promotes lesions and tumor growth in bone

Because of the loss of TβRII expression in CAOBs in patient samples, we focused on dissecting the mechanism by which this loss contributes to PCa bone metastasis. We used the *Tgfbr2*^{Col1CreERT} KO mouse model, which has a loss of TβRII in osteoblasts.

First, the bone lesion differences in *Tgfbr2*^{Col1CreERT} KO vs *Tgfbr2*^{FloxE2} were further analyzed using three-dimensional microCT. We found a significant decrease in the bone volume/tissue volume (BV/TV) ratio and trabecular number (Tb.N), as well as increased trabecular separation (Tb.Sp) in the PC3-injected *Tgfbr2*^{Col1CreERT} KO mice tibiae (**Figure 3A**). The trabecular thickness (Tb.Th) and bone mineral density (BMD) values were not significantly different (data not shown).

To further explore the cellular events affected by the loss of TβRII in osteoblasts, we performed IHC analysis of CD31 for angiogenesis and of phosphorylated histone H3 (p-HH3) for proliferation, immunofluorescence (IF) for GFP and alpha-smooth muscle actin (α-SMA), and bone histomorphometry analyses of osteoclasts and osteoblasts in PC3-injected tibiae. Relative to the *Tgfbr2*^{FloxE2} mice, *Tgfbr2*^{Col1CreERT} KO mice had significant increases in tumor cell proliferation ($P < 0.01$, 1.7-fold increase in positive IHC staining of PCa cells for p-HH3); microvessel density ($P < 0.05$, 2.2-fold increase in CD31-positive microvessels); osteoclastogenesis ($P < 0.05$, 5.2-fold increase in tartrate-

resistant acid phosphatase (TRAP) staining of osteoclasts); and CAF formation (P <0.05, 3.1 fold increase in α-SMA staining cells) (**Figure 3B-E**). However, there was no significant difference in osteoblastogenesis (P = 0.09, data not shown), although a trend of decrease in *Tgfbr2*^{Col1CreERT} KO relative to *Tgfbr2*^{FloxE2} mice was noticed. These data suggest that loss of TGF-β signaling in osteoblasts promotes PCa-induced bone lesions by increasing tumor cell proliferation, angiogenesis, CAF number, and osteoclastogenesis.

Tgfbr2^{Col1CreERT} KO increased bFGF expression and thus promoted PC3 bone metastasis

To identify the downstream mediator for loss of osteoblast-specific TGF-β signaling, we used cytokine arrays to compare the expression profiles of cytokines and growth factors between the PC3-injected tibiae from *Tgfbr2*^{FloxE2} and *Tgfbr2*^{Col1CreERT} KO mice (data not shown). bFGF was one of the up-regulated factors (P < 0.05) confirmed by enzyme-linked immunosorbent assay (ELISA), and it was significantly increased in PC3-injected tibiae from *Tgfbr2*^{Col1CreERT} KO mice relative to those from *Tgfbr2*^{FloxE2} or PBS-injected *Tgfbr2*^{Col1CreERT} KO mice (**Figure 4A**). Furthermore, a significant increase of mouse (but not human) bFGF mRNA was detected only in the PC3-injected *Tgfbr2*^{Col1CreERT} KO mice tibiae (P < 0.05) (**Figure 4B−D**). Increased bFGF protein expression was detected prominently in CAOBs by IHC staining, which is in line with the qRT-PCR analysis (**Figure 4E**). These results suggest that bFGF was increased in CAOBs that had lost TβRII, which are associated with increases in PC3 bone lesions.

We performed rescue experiments to determine the functional effectiveness of bFGF in mediating the loss of TβRII in PCa bone lesion development (**Figure 5A)**. The bone lesions in IgG-treated *Tgfbr2*^{Col1CreERT} KO mouse tibiae (KO_IgG) increased at a significantly faster rate than the lesions in vehicle-treated *Tgfbr2*^{FloxE2} mouse tibiae (Flox_BSA), and they had significantly greater total area by week 3. Moreover, the Ab-bFGF treatment significantly decreased the rate at which PC3 bone lesions developed in the *Tgfbr2*^{Col1CreERT} KO mouse tibiae, by approximately 0.017 cm^3 per week (KO_Ab group versus KO_IgG group). There was no difference in bone lesion development rate in mice from the KO_Ab group and FLox_BSA groups. The recombinant bFGF treatment significantly increased the rate at which PC3 bone lesions developed in *Tgfbr2*^{FloxE2} mouse tibiae by about 0.012 cm^3 per week (Flox_bFGF versus Flox_BSA). There was no difference of bone lesion development rate in mice from Flox_bFGF group and KO_IgG group (**Figure 5B&C**). These rescue experiments showed that bFGF was mediating the increased bone lesions by loss of TβRII in osteoblasts.

bFGF can bind four different FGF receptors, FGFR1-4, to initiate downstream signaling (19). We found that the expressions of FGFR1 and FGFR4 correlated with changes in PC3 bone lesions, i.e., both were lower in the KO_Ab group relative to the KO_IgG, and higher in the Flox_bFGF group relative to the Flox_BSA (**Figure 5D**). Furthermore, we observed the rescue of PC3 tumor cell proliferation, CAF formation, and angiogenesis by bFGF, as indicated by increases of p-HH3-positive, α-SMA-positive cells and of CD31 microvessel density in the Flox_bFGF group relative to the Flox_BSA group, respectively. Furthermore, PC3 tumor cell proliferation, microvessel density, and CAF formation decreased in the KO_Ab group relative to the KO_IgG group (**Figure 5D,**

E). Together, these data suggest that bFGF is one of the downstream functional mediators of osteoblast-specific TGF-β signaling loss in PCa bone lesion development, possibly by promotion of PCa tumor cell proliferation and angiogenesis.

bFGF promoted osteoclastogenesis and inhibited osteoblastogenesis but did not directly affect PC3 cell proliferation *in vitro*

We tested bFGF effects on osteoclast and osteoblast proliferation or differentiation to delineate the effects of bFGF on cells involved in PC3 bone lesion development and to determine whether the role of bFGF depended on paracrine or autonomous TGF-β signaling. Osteoclasts were differentiated from the bone marrow cells of $Tgfbr2^{Col1CreERT}$ KO mice or $Tgfbr2^{FloxE2}$ mice. We found that cells exposed to bFGF had a dose-dependent, significant increase in osteoclastogenesis. Mature osteoclasts were determined by TRAP-positive staining and the presence of at least three nuclei per cell (**Figure 6A**). No differences in osteoclastogenesis between $Tgfbr2^{FloxE2}$ and $Tgfbr2^{Col1CreERT}$ KO mice were found with or without bFGF treatment. This indicated that the paracrine osteoblast TGF-β signaling was not required for either osteoclastogenesis, or the bFGF-stimulated osteoclastogenesis.

For the osteoblast study, adeno-Cre or -GFP viruses were used to infect bone marrow cells from $Tgfbr2^{FloxE2}$ mice to generate control osteoblasts (OB_Flox) or *Tgfbr2* KO osteoblasts (OB_KO). We found that bFGF inhibited osteoblastogenesis ($P < 0.001$, **Figure 6B**). Osteoblasts were identified by ALP staining. No significant differences in osteoblastogenesis were found between OB_Flox and OB_KO cells with or without bFGF treatments, suggesting that cell autonomous TGF-β signaling did not significantly affect osteoblastogenesis or its inhibition by bFGF.

Furthermore, we found that bFGF had no direct effect on PC3 cell proliferation at the doses (0.1 to 5.0 ng/ml) and time courses (24 to 48 h) examined (**Figure 6C**). Thus, our data suggest that in PC3-induced bone lesion development, bFGF is the downstream mediator of osteoblast-specific TGF-β signaling and that TGF-β signaling does not have a positive or a negative feedback effect on the function of bFGF. Taken together, this suggests that bFGF directly promotes osteoclastogenesis and inhibits osteoblastogenesis, but it does not directly promote PC3 tumor cell proliferation.

bFGF was up-regulated by PC3-derived PTHrP through the increased PTH1R

To understand the mechanism of the increase of bFGF in osteoblasts with loss of TGF-β signaling, we co-cultured PC3 cells with OB_Flox or OB_KO cells. bFGF proteins were increased concurrently with the increase of PTH1R and pCREB (**Figure 7A**). This is consistent with previous reports that loss of TβRII increases PTH1R due to the disruption of PTH-induced endocytosis of PTH1R/TβRII (20). It is likely that PTHrP secreted from PC3 cells (12,21-23) induces PTH1R/TβRII endocytosis in this scenario. To test this, neutralizing antibody of PTHrP was added to the co-cultures. The increases of bFGF, PTH1R and pCREB were abolished (**Figure 7A)**. We therefore propose a model (**Figure 7B)** that PCa-derived PTHrP binds to the increased PTH1R, thus activating downstream PKA signaling, which increases pCREB expression, thus up-regulatings bFGF.

Further, to dissect the downstream signaling of the bFGF pathway involved in PC3 bone lesion development, we performed western blots on well-known downstream factors of bFGF. We found only Stat3 and p-Stat3 to be slightly increased in OB_KO co-

culture with PC3 cells, relative to OB_Flox PC3 co-culture (**Supplemental Figure 5**). pERK and pAKT were not changed. These data suggest that the downstream signaling of bFGF in CAOBs possibly occurred through PIK3/Stat3, but not through PIK3/AKT or MAPK/ERK.

Discussion

This is the first study that reports a loss of TβRII in the cancer-associated osteoblasts of PCa patient bone metastasis. Using mouse models with a deletion of *Tgfbr2* specifically in osteoblasts or osteoclasts, our study revealed the cell-specific role of TGF-β signaling in the bone microenvironment and its effect on PCa bone lesion development. Our data suggest that TGF-β signaling in osteoblasts inhibited, but in osteoclasts stimulated, PCa bone metastasis. bFGF was identified as a key mediator in the promotion of PCa bone metastasis. Furthermore, the increase of bFGF in CAOBs that had lost TβRII correlated with the increases of PTH1R and pCREB, suggesting that bFGF was increased at least partially by the activation of PTH1R. Our study is the first to determine the mechanism by which osteoblast-specific TGF-β signaling in the bone microenvironment moderates PCa bone metastasis through coordination, at least in part, with PTH1R/PKA signaling. We also found bFGF to be a potential therapeutic target for PCa bone metastasis.

We confirmed the specificity of this osteoblast-specific TGF-β signaling effect using the $Tgfbr2^{OcnCre}$ mouse model, which has loss of TGF-β signaling in mature osteoblasts. $Tgfbr2^{OcnCre}$ KO mice had a significant increase of trabecular bone and bone mineral density ($P < 0.001$) relative to $Tgfbr2^{flox}$ mice, as reported by others as well (20). PC3-induced bone lesions from cardiac injections were also significantly decreased in $Tgfbr2^{OcnCre}$ vs $Tgfbr2^{flox}$ mice (data not shown).

bFGF has been reported to stimulate the proliferation of the mesenchymal cells such as CAFs and of endothelial cells in angiogenesis, both of which are known to contribute to tumor growth (24,25). Because CAFs and angiogenesis were increased in

PC3 tumors from *Tgfbr2*^{Col1CreERT} KO tibiae and were also altered in the rescue studies, these data suggest that bFGF indirectly induced tumor cell proliferation through increases in CAFs and angiogenesis. Furthermore, *in vitro* osteoclastogenesis or osteoblastogenesis was not significantly different between *Tgfbr2*^{FloxE2} and *Tgfbr2*^{Col1CreERT} KO mice; this is consistent with the normal bone phenotype observed in these mice. On the other hand, bFGF directly stimulated osteoclastogenesis but inhibited osteoblastogenesis, independent of the paracrine or autonomous TGF-β signaling in osteoblasts. Because tumor-associated osteoclasts or CAOBs were too few to be counted at the study end point, we could not confirm this in our *in vivo* rescue studies. Taken together, effects of bFGF on PCa bone metastasis are likely two-fold: indirect promotion of tumor growth and direct stimulation of bone resorption.

bFGF belongs to the fibroblast growth factor family, which consists of 22 members. These ligands bind to the four FGF receptors, which are receptor tyrosine kinases (RTKs) (19,26). bFGF can bind to all four FGFRs. In our study, FGFR1 and FGFR4, but not FGFR2 or FGFR3, were detected using western blots in PCa-injected mouse tibiae. This is consistent with previous findings that FGFR1 is expressed at high levels in the majority of PCa tumors, while FGFR4 is strongly expressed in high grade PCa (27). It might be that bFGF positively regulates the expression of FGFR1 and FGFR4. However, it is possible that the expression changes of FGFR1 and FGFR4 are merely a reflection of tumor growth in tibiae. The downstream signaling pathways of FGFs include MAPK, PI3K, and PKC (28). Dysregulation of FGFs and FGFRs have been linked to the initiation, progression, and metastasis of many cancers, including PCa (26,29-32). Blocking this axis by targeting the receptors has been actively investigated

preclinically and clinically for many cancers and for bone metastasis, and most of these therapeutic efforts have targeted FGFRs (25,32-35). However, study showed that most cancer cells could be rescued from anti-RTK drug sensitivity by simply exposing them to one or more RTK ligands, hepatocyte growth factor (HGF), FGF and neuregulin 1 were the most broadly active ligands (36). These RTK ligands such as FGF could be produced by the cancer-associated stroma as we demonstrated here. In addition, despite some commonalities, each of the ligands has a unique effect and regulatory function. Therefore, it is crucial to understand the regulation of FGFs in cancer, including PCa bone metastasis.

Recently, TβRII was reported to phosphorylate, bind, and form a complex with PTH1R for endocytosis, a process that down-regulates the downstream Smad and PKA/cAMP signaling pathways in osteoblasts (20). Thus, loss of TβRII increases PTH1R availability in the plasma membrane. Ligands such as PTHrP from the PCa cells can then bind to PTH1R to activate downstream PKA/cAMP signaling. bFGF is known to be directly stimulated by PTH through downstream PKA/cAMP signaling (37) and CREB-binding elements in the bFGF gene (*FGF2*) promoter, which have been identified by ChIP sequencing. Therefore, we propose the working model that PC3-derived PTHrP binds PTH1R, which is increased because of the loss of TβRII, and activates the downstream PKA/cAMP/pCREB pathway to stimulate bFGF. bFGF, in turn, stimulates osteoclastogenesis, inhibits osteoblastogenesis, and promotes PCa growth through inducing the formation of CAFs and angiogenesis. bFGF is expressed primarily by cells of the mesenchymal lineage and our results showed that PC3-injected *Tgfbr2*^{Col1CreERT} KO tibiae had increased bFGF production from osteoblasts but not from PCa cells.

Furthermore, PTHrP is highly expressed in bone metastatic cancer cells such as PC3 cells, as reported by others (12,21), and the increases of bFGF, pCREB and PTH1R in co-cultured OB_KO cells (relative to OB_Flox) were abolished by blocking PTHrP. This model is further supported by our PCa patient tissue microarray data, which showed higher TβRII expression in PCa cells from bone metastases relative to visceral metastases. TGF-β signaling is known to induce the expression of PTHrP in cancer cells (38-40), thus, in bone metastasis, the increased PTHrP from PCa cells further activates the elevated PTH1R in CAOBs (due to loss of TβRII) to promote bone lesion development.

Because cancer-associated osteoclasts were identified only in a few samples of the PCa bone metastasis examined, the overall expression of TβRII could not be determined. However, in a few cancer-associated osteoclasts, TβRII was indeed expressed (data not shown). In addition, our current studies of *Tgfbr2*^{LysMCre} KO mice and a previous study of breast cancer patient bone metastasis (20) suggest that TβRII is expressed in cancer-associated osteoclasts and that TGF-β signaling in osteoclasts promotes bone metastasis. The lack of cancer-associated osteoclasts, in our opinion, reflects that the majority of PCa bone metastasis is ostoeblastic. However, bone lesion phenotypes were not provided in the TMAs studied here.

We also recognize that there is no available mouse model of PCa that simulates bone metastasis from the orthotopic site. Thus, this study is limited to the last step of bone metastasis, i.e., bone lesion development caused by the interactions between PCa cells and the bone microenvironment. PC3 and DU145 are castration-resistant PCa cells, and the androgen receptor (AR) is commonly undetectable in them. Thus, using these

cells represents the biology of 10-15% PCa patients. We also recognize that bFGF may not be the only FGF having its expression altered by the *Tgfbr2*^{Col1CreERT} KO, because other FGFs were not included in the cytokine array used for this study. ENCODE data showed CREB-binding sites in all 22 FGF genes, indicating other FGFs are probably also up-regulated by increased PKA/cAMP signaling. Therefore, in the future, we will identify other FGFs and block them along with bFGF, aiming for complete inhibition of PCa bone lesion development.

In summary, our study revealed that loss of TβRII increased the availability of PTH1R, resulting in loss of TGF-β signaling and increased PTH1R/PKA signaling. Therefore, PCa bone metastasis was promoted by the coordination of these two signaling pathways, and bFGF was one of the functional effectors that can be targeted. As a translation contribution, our research suggests that clinical treatments positively affecting TβRII expression in CAOBs could benefit patients, but those associated with a decrease of TβRII expression in CAOBs should be discouraged.

Materials and methods

Cells, animals, and reagents

The PCa cell lines PC3 and DU145 were purchased from the American Type Culture Collection (ATCC**;** Manassas, VA) and were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS).

Tgfbr2^{FloxE2} mice (16) were bred with *Col1a2*^{CreERT} (17), *Lysm*^{Cre} (18), or *Ocn*^{Cre} (41) mice to generate *Tgfbr2*^{Col1CreERT}, *Tgfbr2*^{LysmCre}, or *Tgfbr2*^{OcnCre} KO mice, respectively. These mice were further bred to either ROSA26 or mT/mG (JAX, 007676) reporter mice for the visualization of Cre activity, and to *Rag-2* (JAX, 008449) KO mice, whose immunodeficiency allows for the inoculation of human cells. All the mice were bred, maintained, and used in this study with approval of the VARI Institutional Animal Care and Use Committee.

Commercially available reagents were purchased. The detailed information, including the doses used in our studies, is listed in **Supplemental Table 2**.

Prostate cancer metastasis tumor microarray

IHC analysis of TβRII was performed on a UWTMA79 prostate cancer metastasis tumor microarray (TMA), which contained samples from 45 cases of bone and visceral metastasis from rapid autopsy (62 visceral and 79 bone metastasis). Detailed information can be found on the PCBN website. The pathologist from our Institute, as well as two individuals from our laboratory, evaluated the staining independently. Scores of 1 to 5 were given based on the intensity of the staining, and 0 was assigned to negative staining. The final scores given to one cell type of each tissue was an average of the scores from

the three cores of each tissue sample. All evaluations were submitted to the Bioinformatics and Biostatistics Core for further analyses before the final conclusion.

Injections and radiographic imaging (14,18)

To generate the *Tgfbr2*^{Col1CreERT} KO mice, Cre-positive male mice at age 4 to 5 weeks were intraperitoneally (*i.p.*) injected with tamoxifen (40 μg/g body weight) for 5 d. At 5 to 7 days after the last injection, the mice were randomly allocated for treatment. The Cre-negative littermates were treated the same as the Cre-positive mice and served as *Tgfbr2*^{FloxE2} control mice. For intratibial injections, one million PC3 or DU145 PCa cells in 10 μl of PBS were injected into the left tibia, and 10 μl of PBS was injected into the contralateral tibia as a control.

Mice were imaged using Bioptics piXarray Digital Specimen Radiography (Faxitron Bioptics) at 2, 3, and 4 weeks post injection for PC3 cells or at 3, 5, and 7 weeks post injection for DU145 cells. Mice that never developed lesions were considered technical injection failures and were excluded from the studies. No data points from mice with successful injections were excluded from the data set. The bone lesions were counted and areas were measured from X-rays using MetaMorph (Molecular Devices, Inc.). All defined regions of interest (ROIs) were analyzed. The cell preparations, injections, imaging, and bone lesion analysis were performed blinded by individuals of our lab.

Mouse tibiae were harvested in 70% ethanol and subjected to microCT scanning and imaging using a SKYSCAN 1172 μCT instrument (Bruker, Ettlingen, Germany). The trabecular ROI extended 8 mm from the subchondral plate in the distal direction in

order to include all potential tumor areas. SkyScan software (DataViewer, CTAn, and CTVox) was used to determine the three-dimensional structural parameters, including BV/TV, Tb.Th, Tb.Sp, Tb.N, BMD, mean total cross-sectional bone perimeter (B.Pm), average object equivalent circle diameter per slice (Av.Obj.ECD), and cross-sectional thickness (Cs.Th). All measured variables in the left tibiae with PCa were normalized to right tibiae.

Histology, histomorphometry, immunohistochemistry, and immunofluorescence

Mouse tibiae were harvested, fixed, processed, sectioned, stained, and analyzed as previously described (18). Serial sections were stained for TRAP and with H&E to confirm the osteoclast and osteoblast identity. The stained sections were subjected to histomorphometry analyses of tumor burden. The numbers of osteoclasts and osteoblasts, as well as osteoclast surfaces, were determined using Bioquant system imaging software 2014 (Nashville, TN). IHC was performed on the other sections to assess the expression of GFP, p-Smad2, CD31, p-HH3, α-SMA, and bFGF. Immunofluorscence was performed on the sections to assess the expression of GFP and α-SMA.

Protein extraction, western blots, and cytokine arrays (18)

Tibiae were harvested and snap-frozen in liquid nitrogen. The frozen tibiae were then homogenized using FastPrep-24 (MP Biomedicals). For western blots, 40 μg of total protein per sample was used. For cytokine arrays, equal amounts of protein per tibia were pooled from three mice (either *Tgfbr2*^{FloxE2} or *Tgfbr2*^{Col1CreERT} KO littermates) 3 weeks after PC3 tibial injection. qRT-PCR was performed using SYBR Supermix (Bio-Rad,

Hercules, CA) on the ABI machine. Primers were custom-synthesized (IDT, Coralville, IA). The sequences of the primers are listed in **Supplemental Table 3**.

Application of neutralizing antibody or recombinant bFGF *in vivo*

Briefly, one day after intratibial injection of PC3 cells, neutralizing bFGF antibody (Ab-bFGF) or control antibody (IgG) was retro-orbitally *i.v.* injected into the *Tgfbr2*^{Col1CreERT} KO mice, and recombinant bFGF or 0.1% BSA vehicle was injected *i.v.* into the *Tgfbr2*^{FloxE2} mice. The drugs and vehicles were administered every other day up to 4 weeks post injection. The host mice were X-rayed for bone lesions weekly; both serum and tibiae were collected at the indicated time points.

Bone marrow differentiation and co-culture with PC3 cells

For osteoblastogenesis, mouse bone marrow cells from *Tgfbr2*^{FloxE2} mice were used. On the third day of culture, these bone marrow cells were infected with either adeno-Cre or adeno-GFP to generate either *Tgfbr2* KO (OB_KO) or *Tgfbr2* Flox (OB_Flox) control cells, respectively. These cells were then further differentiated for another 7 d in α-MEM medium supplemented with 10% FBS and 50 μg/ml of ascorbic acid. The media was changed every 3 d until the end of the experiments. Alkaline phosphatase (ALP) staining was performed. For the co-culture experiment, PC3 cells were plated in the insert the second day after plating the bone marrow cells. The PTHrP neutralizing antibodies were added at the same day with PC3 cells at a dose of 1μg/ml, and changed every day.

For the osteoclastogenesis, the bone marrow cells from either *Tgfbr2*^{Col1CreERT} KO or *Tgfbr2*^{FloxE2} mice were counted and cultured in α-MEM with 30 ng/ml macrophage colony-stimulating factor (MCSF) on the first day. On the second day, the non-adherent cells were collected and re-plated with αMEM [10% FBS + 30 ng/ml receptor activator of nuclear factor kappa-B ligand (RANKL) + 30 ng/ml MCSF]. The medium was then changed every 3 d until day 7 or 8. TRAP staining was performed at the end points.

Statistical analysis

Clinical data were first input using a random forest iterated 5 times, with 300 trees each. Then, LASSO (least absolute shrinkage and selection operator) analyses were used to explore any associations between clinical data and cell-specific TβRII expression in PCa patient bone metastasis tissues. To test for differences in the proportion of metastatic tissues that completely lost TβRII expression, logistic mixed-effects models were used. Longitudinal bone lesion data were analyzed via linear mixed-effect model. Linear contrasts with a false discovery rate correction were used to test for significant differences in bone lesion growth rates, and bootstrap hypothesis testing with 1000 resampled data sets was used to test for differences in total lesion area between groups at specific time points. For experiments without repeated measures, data were analyzed via two-way ANOVA when there were two independent variables; otherwise, a two-tailed Student's *t*-test was used. Normality assumptions were assessed visually via QQ-plots; no concerning deviations were detected. The incidence of bone lesions was analyzed via log-rank tests. *t*-tests and ANOVAs were done via Graphpad and all other analyses via R v 3.2.2. For all analyses, $P < 0.05$, two-sided was considered significant.

Study approval

Animal studies were approved by VARI Institutional and Department of Defense Prostate Cancer Research Program (DOD PCRP) Animal Care and Use Committees.

Author contributions

Conception and design, X. Meng, X. Li; performing experiments and acquisition of data, X. Meng, P. Daft, A. Vander Ark, J. Wang, X. Li; analysis and interpretation of data (e.g. pathological scoring, statistical analysis), X. Meng, Z. Madaj, G. Hostetter, X. Li; writing the manuscript, X. Meng, X. Li; study supervision, X. Li.

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References

1. Coleman RE. Clinical features of metastatic bone disease and risk of skeletal morbidity. Clin Cancer Res 2006;12(20 Pt 2):6243s-49s.
2. McCauley LK, Martin TJ. Twenty-five years of PTHrP progress: from cancer hormone to multifunctional cytokine. J Bone Miner Res 2012;27(6):1231-9.
3. Liao J, Li X, Koh AJ, Berry JE, Thudi N, Rosol TJ, et al. Tumor expressed PTHrP facilitates prostate cancer-induced osteoblastic lesions. International journal of cancer 2008;123(10):2267-78.
4. Bryden AA, Islam S, Freemont AJ, Shanks JH, George NJ, Clarke NW. Parathyroid hormone-related peptide: expression in prostate cancer bone metastases. Prostate Cancer Prostatic Dis 2002;5(1):59-62.
5. Bryden AA, Hoyland JA, Freemont AJ, Clarke NW, George NJ. Parathyroid hormone related peptide and receptor expression in paired primary prostate cancer and bone metastases. Br J Cancer 2002;86(3):322-5.
6. Mundy GR. Mechanisms of bone metastasis. Cancer 1997;80(8 Suppl):1546-56.
7. Padua D, Massague J. Roles of TGFbeta in metastasis. Cell Res 2009;19(1):89-102.
8. Buijs JT, Stayrook KR, Guise TA. The role of TGF-beta in bone metastasis: novel therapeutic perspectives. Bonekey Rep 2012;1:96.
9. Li J, Karaplis AC, Huang DC, Siegel PM, Camirand A, Yang XF, et al. PTHrP drives breast tumor initiation, progression, and metastasis in mice and is a potential therapy target. J Clin Invest 2011;121(12):4655-69.

10. Mundy GR. Metastasis to bone: causes, consequences and therapeutic opportunities. Nat Rev Cancer 2002;2(8):584-93.
11. Roodman GD. Mechanisms of bone metastasis. N Engl J Med 2004;350(16):1655-64.
12. Guise TA, Yin JJ, Taylor SD, Kumagai Y, Dallas M, Boyce BF, et al. Evidence for a causal role of parathyroid hormone-related protein in the pathogenesis of human breast cancer-mediated osteolysis. J Clin Invest 1996;98(7):1544-9.
13. Li X, Placencio V, Iturregui JM, Uwamariya C, Sharif-Afshar AR, Koyama T, et al. Prostate tumor progression is mediated by a paracrine TGF-beta/Wnt3a signaling axis. Oncogene 2008;27(56):7118-30.
14. Li X, Sterling JA, Fan KH, Vessella RL, Shyr Y, Hayward SW, et al. Loss of TGF-beta responsiveness in prostate stromal cells alters chemokine levels and facilitates the development of mixed osteoblastic/osteolytic bone lesions. Mol Cancer Res 2012;10(4):494-503.
15. Placencio VR, Sharif-Afshar AR, Li X, Huang H, Uwamariya C, Neilson EG, et al. Stromal transforming growth factor-beta signaling mediates prostatic response to androgen ablation by paracrine Wnt activity. Cancer Res 2008;68(12):4709-18.
16. Chytil A, Magnuson MA, Wright CV, Moses HL. Conditional inactivation of the TGF-beta type II receptor using Cre:Lox. Genesis 2002;32(2):73-5.
17. Zheng B, Zhang Z, Black CM, de Crombrugghe B, Denton CP. Ligand-dependent genetic recombination in fibroblasts : a potentially powerful

technique for investigating gene function in fibrosis. Am J Pathol 2002;160(5):1609-17.

18. Meng X, Vander Ark A, Lee P, Hostetter G, Bhowmick NA, Matrisian LM, et al. Myeloid-specific TGF-beta signaling in bone promotes basic-FGF and breast cancer bone metastasis. Oncogene 2016;35(18):2370-8.

19. Powers CJ, McLeskey SW, Wellstein A. Fibroblast growth factors, their receptors and signaling. Endocr Relat Cancer 2000;7(3):165-97.

20. Qiu T, Wu X, Zhang F, Clemens TL, Wan M, Cao X. TGF-beta type II receptor phosphorylates PTH receptor to integrate bone remodelling signalling. Nat Cell Biol 2010;12(3):224-34.

21. Sterling JA, Oyajobi BO, Grubbs B, Padalecki SS, Munoz SA, Gupta A, et al. The hedgehog signaling molecule Gli2 induces parathyroid hormone-related peptide expression and osteolysis in metastatic human breast cancer cells. Cancer Res 2006;66(15):7548-53.

22. Deftos LJ, Barken I, Burton DW, Hoffman RM, Geller J. Direct evidence that PTHrP expression promotes prostate cancer progression in bone. Biochem Biophys Res Commun 2005;327(2):468-72.

23. Iwamura M, Abrahamsson PA, Foss KA, Wu G, Cockett AT, Deftos LJ. Parathyroid hormone-related protein: a potential autocrine growth regulator in human prostate cancer cell lines. Urology 1994;43(5):675-9.

24. Kwabi-Addo B, Ozen M, Ittmann M. The role of fibroblast growth factors and their receptors in prostate cancer. Endocr Relat Cancer 2004;11(4):709-24.

25. Bono F, De Smet F, Herbert C, De Bock K, Georgiadou M, Fons P, et al. Inhibition of tumor angiogenesis and growth by a small-molecule multi-FGF receptor blocker with allosteric properties. Cancer Cell 2013;23(4):477-88.
26. Turner N, Grose R. Fibroblast growth factor signalling: from development to cancer. Nat Rev Cancer 2010;10(2):116-29.
27. Sahadevan K, Darby S, Leung HY, Mathers ME, Robson CN, Gnanapragasam VJ. Selective over-expression of fibroblast growth factor receptors 1 and 4 in clinical prostate cancer. J Pathol 2007;213(1):82-90.
28. Goetz R, Mohammadi M. Exploring mechanisms of FGF signalling through the lens of structural biology. Nat Rev Mol Cell Biol 2013;14(3):166-80.
29. Murphy T, Darby S, Mathers ME, Gnanapragasam VJ. Evidence for distinct alterations in the FGF axis in prostate cancer progression to an aggressive clinical phenotype. J Pathol 2010;220(4):452-60.
30. Valta MP, Tuomela J, Bjartell A, Valve E, Vaananen HK, Harkonen P. FGF-8 is involved in bone metastasis of prostate cancer. International journal of cancer 2008;123(1):22-31.
31. Yang F, Zhang Y, Ressler SJ, Ittmann MM, Ayala GE, Dang TD, et al. FGFR1 is essential for prostate cancer progression and metastasis. Cancer Res 2013;73(12):3716-24.
32. Wan X, Corn PG, Yang J, Palanisamy N, Starbuck MW, Efstathiou E, et al. Prostate cancer cell-stromal cell crosstalk via FGFR1 mediates antitumor activity of dovitinib in bone metastases. Sci Transl Med 2014;6(252):252ra122.

33. Li F, Huynh H, Li X, Ruddy DA, Wang Y, Ong R, et al. FGFR-Mediated Reactivation of MAPK Signaling Attenuates Antitumor Effects of Imatinib in Gastrointestinal Stromal Tumors. Cancer Discov 2015;5(4):438-51.
34. Azuma K, Kawahara A, Sonoda K, Nakashima K, Tashiro K, Watari K, et al. FGFR1 activation is an escape mechanism in human lung cancer cells resistant to afatinib, a pan-EGFR family kinase inhibitor. Oncotarget 2014;5(15):5908-19.
35. Hagel M, Miduturu C, Sheets M, Rubin N, Weng W, Stransky N, et al. First Selective Small Molecule Inhibitor of FGFR4 for the Treatment of Hepatocellular Carcinomas with an Activated FGFR4 Signaling Pathway. Cancer Discov 2015;5(4):424-37.
36. Wilson TR, Fridlyand J, Yan YB, Penuel E, Burton L, Chan E, et al. Widespread potential for growth-factor-driven resistance to anticancer kinase inhibitors. Nature 2012;487(7408):505-U1652.
37. Hurley MM, Tetradis S, Huang YF, Hock J, Kream BE, Raisz LG, et al. Parathyroid hormone regulates the expression of fibroblast growth factor-2 mRNA and fibroblast growth factor receptor mRNA in osteoblastic cells. J Bone Miner Res 1999;14(5):776-83.
38. Lindemann RK, Ballschmieter P, Nordheim A, Dittmer J. Transforming growth factor beta regulates parathyroid hormone-related protein expression in MDA-MB-231 breast cancer cells through a novel Smad/Ets synergism. J Biol Chem 2001;276(49):46661-70.

39. Yin JJ, Selander K, Chirgwin JM, Dallas M, Grubbs BG, Wieser R, et al. TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. J Clin Invest 1999;103(2):197-206.
40. Kakonen SM, Selander KS, Chirgwin JM, Yin JJ, Burns S, Rankin WA, et al. Transforming growth factor-beta stimulates parathyroid hormone-related protein and osteolytic metastases via Smad and mitogen-activated protein kinase signaling pathways. J Biol Chem 2002;277(27):24571-8.
41. Zhong Z, Zylstra-Diegel CR, Schumacher CA, Baker JJ, Carpenter AC, Rao S, et al. Wntless functions in mature osteoblasts to regulate bone mass. Proc Natl Acad Sci U S A 2012;109(33):E2197-204.
42. Szasz AM, Nyirady P, Majoros A, Szendroi A, Szucs M, Szekely E, et al. beta-catenin expression and claudin expression pattern as prognostic factors of prostatic cancer progression. BJU Int 2010;105(5):716-22.

Figures

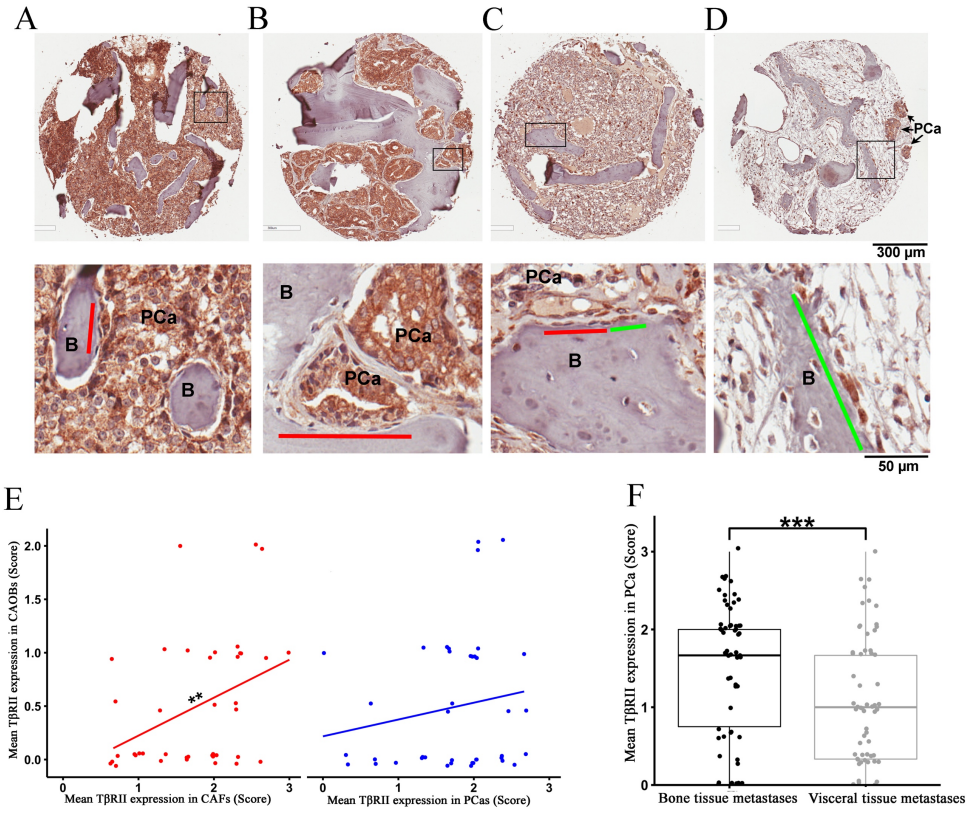

Figure 1. TβRII expression in PCa patient bone metastasis tissues. Immunohistochemical (IHC) analysis of TβRII on a PCa patient bone metastasis tissue microarray containing 79 PCa bone metastasis and 62 visceral metastasis tissues (each having three cores) plus normal tissues as controls. **A-D)** IHC of TβRII showed positive (brown) staining in PCa cells in almost all samples examined. Loss of TβRII in all CAOBs was observed in 21 out of 40 tissues in which osteoblasts were identified (**A&B**, indicated by red lines); the estimated probability of this loss was 54.2% (95% CI, 36.3% to 68.2%). A mixture of both loss and expression of TβRII among CAOBs was observed in 16 out of 40 tissues (**C**, indicated by red and green lines); the estimated probability of this mixed expression was 40.5% (mixed-effects logistic regression, 95% CI, 27.6% to 54.4%). (scale bars: upper images, 300 μm; lower images, 50 μm) Expression of TβRII in all CAOBs was observed only in 3 out of 40 tissues (**D**, indicated by green line); the estimated probability of this expression was 4.5% (95% CI, 0.6% to 13.4%). **E)** Association analysis of cell-specific TβRII expression. TβRII expression in CAOBs was positively correlated with expression in CAFs, but not with expression in PCa cells (linear mixed-effects model, ** $P < 0.01$). **F)** TβRII expression in PCa cells of bone metastasis tissues was significantly higher than in visceral tissues (*t* test, two-tailed, *** $P < 0.001$).

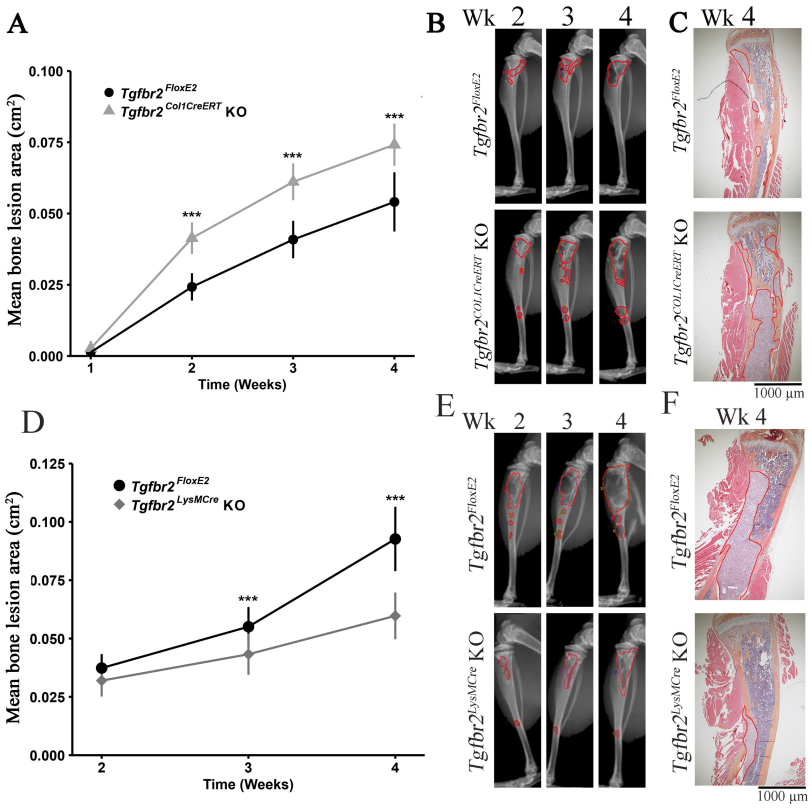

Figure 2. Effects of *Tgfbr2*^{Col1CreERT} and *Tgfbr2*^{LysMCre} KO on PC3-induced bone lesion development.

A and **D)** Mean bone lesion area in PC3-injected mouse tibiae. The bone lesions were imaged by weekly X-rays, from which the bone lesion areas were measured using Metamorph software. **A**) The average total area of PC3 osteolytic lesions in *Tgfbr2*^{Col1CreERT} KO mice increased by 0.01 cm^2 per week more than lesions in *Tgfbr2*^{FloxE2} mice. Significant differences in bone lesion development were found between *Tgfbr2*^{FloxE2} and *Tgfbr2*^{Col1CreERT} KO mice (linear mixed-effects model with a random intercept, *** $P < 0.001$, $n \geq 9$). **D)** The average total area of PC3 osteolytic lesions in *Tgfbr2*^{LysMCre} KO mice was inhibited by 0.01 cm^2 per week relative to *Tgfbr2*^{FloxE2} mice. Significant differences in bone lesion development between *Tgfbr2*^{FloxE2} and *Tgfbr2*^{LysMCre} KO mice were found (linear mixed-effects model with a random intercept, *** $P < 0.001$, $n \geq 9$). **B and E)** Representative X-ray images of the osteolytic bone lesions at each time point. The circled lines show the regions of interest (42) that were measured and analyzed using Metamorph Software. **C and F)** Representative H&E staining of the tibiae. The red outlines indicate the PC3 tumor growth in the bone marrow (scale bar, 100 µm).

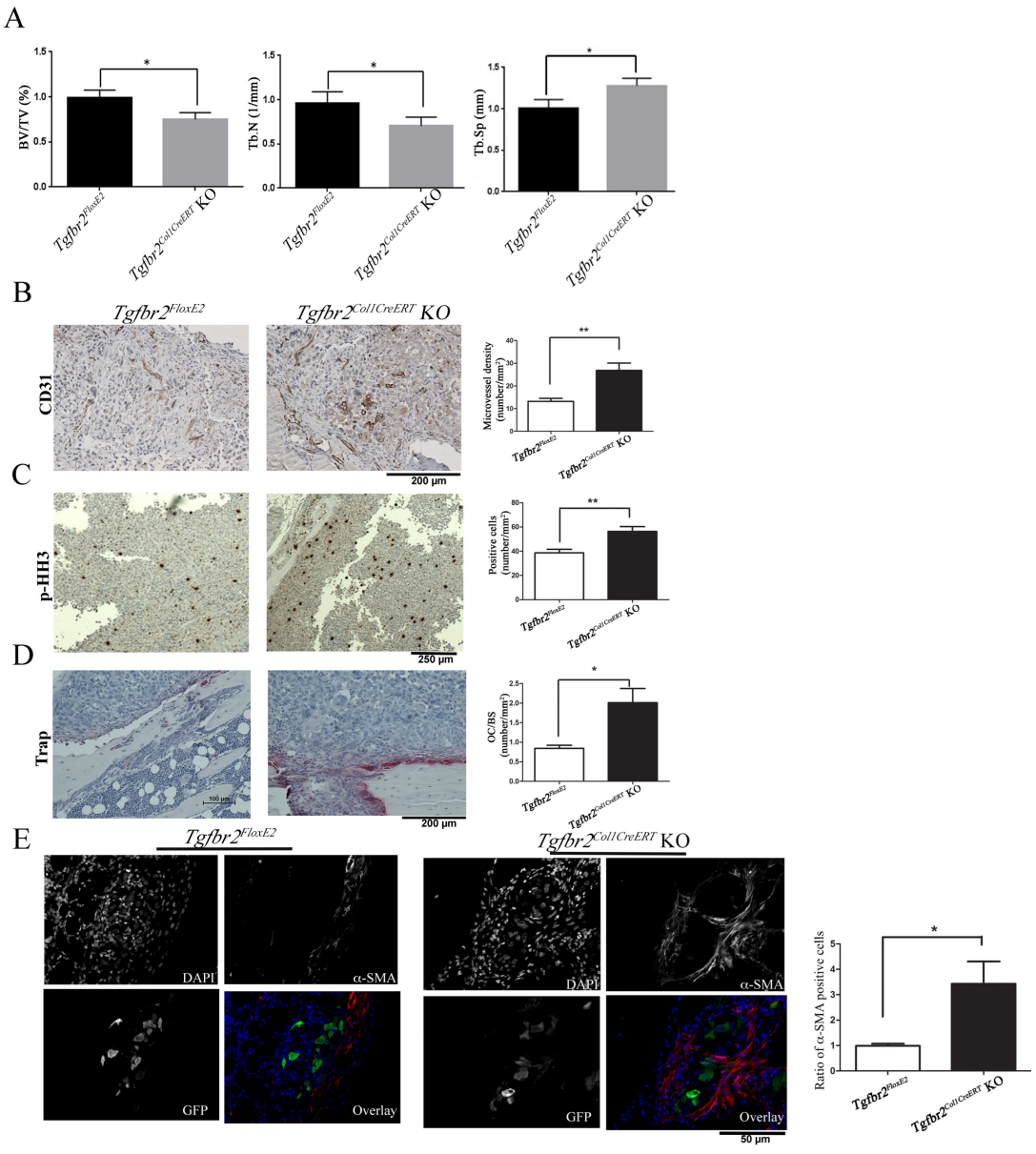

Figure 3. *Tgfbr2*^{Col1CreERT} KO promoted osteolytic lesion development and tumor growth.

A) Quantitative µCT analyses. Significant decreases in trabecular bone volume/total bone volume (BV/TV), trabecular bone number (Tb.N), but increased trabecular bone separation (Tb.Sp) were found in *Tgfbr2*^{Col1CreERT} KO tibiae, relative to tibiae from *Tgfbr2*^{FloxE2} mice. All data were normalized to the contralateral tibia. (*t* test, two-tailed, * $P \leq 0.05$, ** $P < 0.01$, $n \geq 3$). Representative IHC staining of **B)** CD31 (2.0-fold increase) (scale bar, 200 µm) and **C)** phosphorylated-histone H3 (P-HH3) (1.46-fold increase) (scale bar, 250 µm). **D)** tartrate-resistant acid phosphatase (TRAP) staining (2.38-fold increase) (scale bar, 200 µm). Quantifications are shown to the right of the image panels. **E)** Representative immunofluorescence and quantification of the α-SMA positive fibroblasts (3.47-fold increase). The data was normalized to the number of GFP-positive PC3 cells (scale bar, 50 µm).

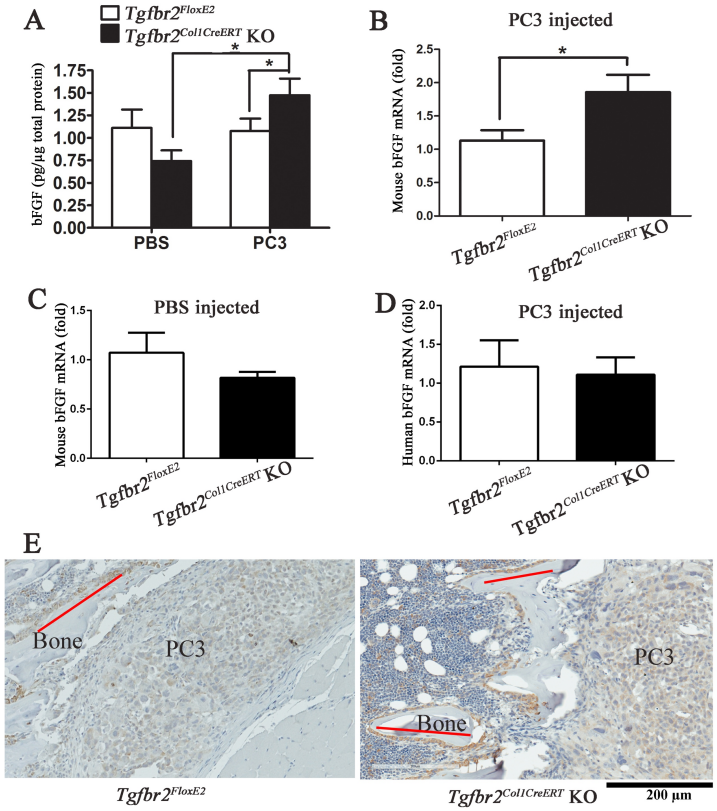

Figure 4. *Tgfbr2*^{Col1CreERT} KO up-regulated bFGF expression in CAOBs of PC3 bone lesions.

A) ELISA quantification. bFGF protein was measured from crushed whole tibiae that had been injected with either PBS or PC3 cells. A significant increase of bFGF protein was detected in PC3-injected tibiae of *Tgfbr2*^{Col1CreERT} KO mice (relative to *Tgfbr2*^{FloxE2} mice; two-way ANOVA, * $P < 0.05$). **B–D)** qRT-PCR analyses of the relative expression of species-specific bFGF mRNA from mouse tibiae. Mouse bFGF mRNA was significantly increased in PC3-injected tibiae of *Tgfbr2*^{Col1CreERT} KO mice (relative to *Tgfbr2*^{FloxE2} mice; *t* test, two-tailed, * $P \leq 0.05$, $n \geq 3$). **E)** Representative IHC images of bFGF expression, which was increased in CAOBs from *Tgfbr2*^{Col1CreERT} KO mice (scale bar: 200 μm).

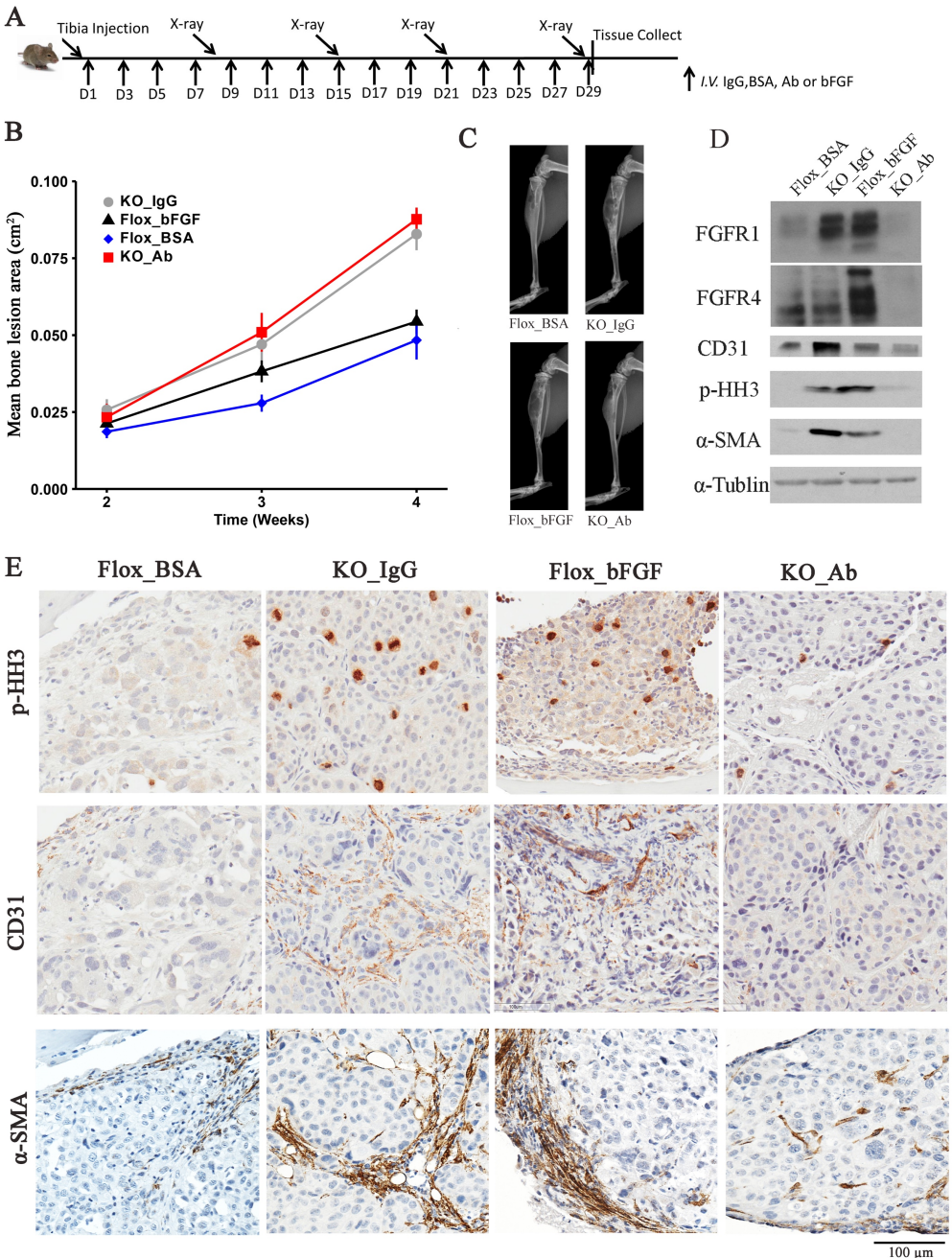

Figure 5. bFGF mediated the increased PC3 bone lesion in *Tgfbr2*^{Col1CreERT} KO mice. **A)** Schedule of the drug treatment, X-ray image acquisition, and end-point collection. **B)** Quantification of mean bone lesion area in PC3-injected mouse tibiae. Significant bone lesion development was found between groups of the following: Flox_BSA vs KO_IgG, Flox_BSA vs Flox_bFGF, and KO_IgG vs KO_Ab (linear mixed-effects model with a random intercept, *** $P < 0.001$, $n \geq 3$). **C)** Representative X-ray images from each group at the final time point. **D)** Western blot analyses from whole tibiae harvested at the final time point. **E)** Representative images of IHC staining of p-HH3, CD31 and α-SMA (scale bar, 100 μm).

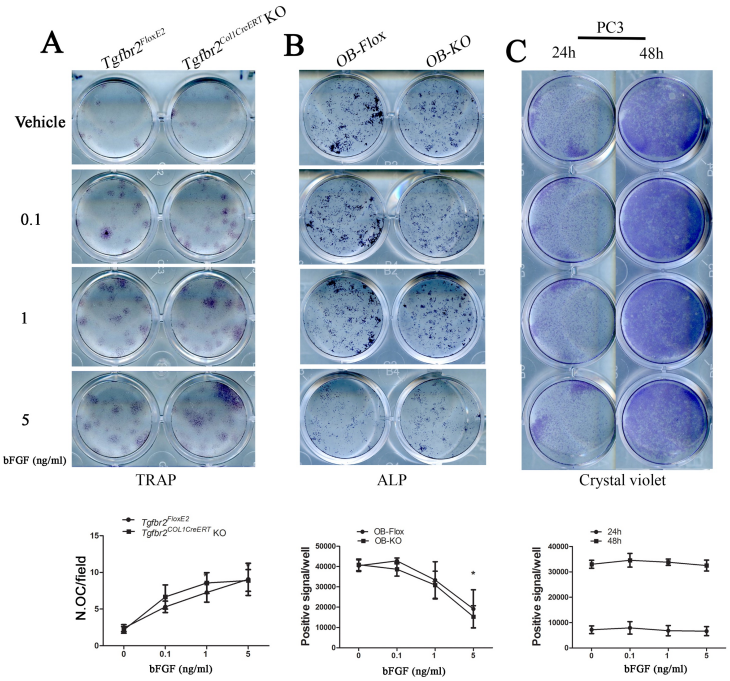

Figure 6. bFGF promoted osteoclastogenesis, inhibited osteoblastogenesis, but had no effect on PC3 proliferation.

Cells were exposed to bFGF at the doses and time points as indicated. **A)** Representative TRAP staining and quantification for osteoclasts from *Tgfbr2*^{FloxE2} and *Tgfbr2*^{Col1CreERT} KO littermates. A dose-dependent increase of osteoclast differentiation by bFGF was observed, but the effects of bFGF were not different between the cell lines. (two-way ANOVA, *** $P \leq 0.001$, $n \geq 3$). **B)** Representative ALP staining and quantification for control osteoblasts (OB-Flox) and osteoblasts with deletion of the *Tgfbr2* gene (OB-KO). A dose-dependent decrease of osteoblast differentiation by bFGF was observed, and the effects of bFGF were not different between the cell lines (two-way ANOVA, *** $P \leq 0.001$, $n \geq 3$). **C)** Representative images of PC3 cells proliferation that were stained with crystal violet. bFGF had no effect on PC3 cell proliferation after 24-h or 48-h treatment with various doses (one way ANOVA, $n \geq 3$).

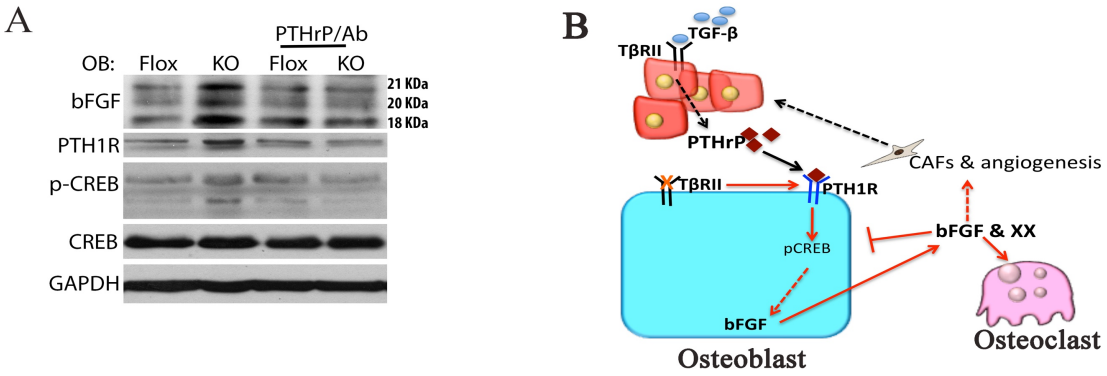

Figure 7. Loss of TβRII resulted an increase of PTH1R through which bFGF was up-regulated in osteoblasts by PC3-derived PTHrP. A) Representative western blots of total protein extracted from osteoblasts that were co-cultured with PC3 cells, n = 5. **B)** Loss of TβRII in cancer-associated osteoblasts (CAOBs) resulted an increase of PTH1R, possibly through disruption of the PTH1R/TβRII endocytosis induced by PCa-derived PTHrP. The increase of PTH1R was bound and activated the downstream PKA/pCREB signaling by PC3-drived PTHrP to up-regulate bFGF. bFGF, in turn, promoted PCa induced-bone lesion development by stimulating the differentiation of cancer-associated osteoclasts (CAOCs), inhibiting the differentiation of CAOBs, and potentially stimulates PCa proliferation through increasing cancer-associated fibroblasts (CAFs) and angiogenesis. In addition, high expression of TβRII in the bone metastatic PCa cells suggested an increased PTHrP expression by TGF-β from the bone microenvironment. Note: solid lines indicate our data and dashed lines indicate published data from others or our hypotheses.